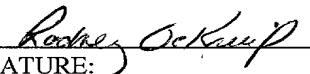


Form PTO-1390 (REV 11-2000)		US DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 4856-CIP
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371			US APPLICATION NO (If known, see 37 CFR 15) 09/914501
INTERNATIONAL APPLICATION NO. PCT/US00/05364	INTERNATIONAL FILING DATE 01 March 2000	PRIORITY DATE CLAIMED 01 March 1999	
TITLE OF INVENTION METHOD OF DIAGNOSING AND MONITORING MALIGNANT BREAST CARCINOMAS			
APPLICANT(S) FOR DO/EO/US STRECKFUS, CHARLES F.; BIGLER, LENORA G.; THIGPEN, JAMES TATE			
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:			
<ol style="list-style-type: none"> 1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371. 2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371. 3. <input type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1). 4. <input checked="" type="checkbox"/> The U.S. has been elected by the expiration of the 19th month from the priority date. 5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)) <ol style="list-style-type: none"> a. <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the international Bureau). b. <input type="checkbox"/> has been transmitted by the International Bureau. c. <input checked="" type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). 6. <input type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)). 7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Articles 19 or 34 (35 U.S.C. 371(c)(3)) <ol style="list-style-type: none"> a. <input checked="" type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau). b. <input checked="" type="checkbox"/> have been transmitted by the International Bureau in English. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input type="checkbox"/> have not been made and will not be made. 8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)). 9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). 10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report Under PCT Article 36 (35 U.S.C. 371(c)(5)). 			
Items 11. to 16. below concern document(s) or information included:			
<ol style="list-style-type: none"> 11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98. 12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. 13. <input checked="" type="checkbox"/> A FIRST preliminary amendment. 14. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment. 15. <input type="checkbox"/> A substitute specification. 16. <input type="checkbox"/> A change of power of attorney and/or address letter. 17. <input type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825. 18. <input type="checkbox"/> A second copy of the published international application under 35 U.S.C. 154(d)(4). 19. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4). 20. <input checked="" type="checkbox"/> Other items of information: Copy of Small Entity Statement for Priority Application and Assertion of Small Entity Status 			

US APPLICATION NO. (if known, see 37 CFR 1.55) 09/914501		INTERNATIONAL APPLICATION NO. PCT/US00/05364		ATTORNEY'S DOCKET NUMBER 4856-CIP	
21. <input checked="" type="checkbox"/> The following fees are submitted: BASIC NATIONAL FEE (37 CFR 1.492(a) (1) - (5)): Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO. \$1000.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO. \$860.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$710.00 <input checked="" type="checkbox"/> International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(I)-(4). \$690.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(I)-(4). \$100.00 ENTER APPROPRIATE BASIC FEE AMOUNT = \$690				CALCULATIONS PTO USE ONLY	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	20-20 =	0	X \$18.00	\$	
Independent claims	3-3 =	0	X \$80.00	\$	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$270.00	\$	
TOTAL OF ABOVE CALCULATIONS =				\$690	
Reduction of 1/2 for filing by small entity, if applicable. A Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).				\$345	
SUBTOTAL =				\$345	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				\$	
TOTAL NATIONAL FEE =				\$345	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property				\$	
TOTAL FEES ENCLOSED =				\$345	
				Amount to be Refunded: \$	
				Charged: \$	
a. <input checked="" type="checkbox"/> A check in the amount of <u>\$345</u> to cover the above fees is enclosed. b. <input type="checkbox"/> Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>18-0882</u> . <u>A duplicate copy of this sheet is enclosed.</u> d. <input type="checkbox"/> Fees are to be charged to a credit card. WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.					
Note: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.					
SEND ALL CORRESPONDENCE TO:					
CUSTOMER No. 22922					
Reinhart, Boerner, Van Deuren, Norris & Rieselbach, s.c. Attn: Linda Gabriel, Docket Clerk 1000 North Water Street, Suite 2100 Milwaukee, WI 53202-0900 414-298-8360			<div style="text-align: center;">  SIGNATURE: </div> <div style="text-align: center;"> Rodney D. DeKruif NAME </div> <div style="text-align: center;"> <u>35.853</u> REGISTRATION NUMBER </div>		

MW\766976

PATENT

I hereby certify that this paper or fee is being deposited with the United States Postal Service as Express Mail No. EL 835272099 US in an envelope addressed to: Box PCT, Assistant Commissioner for Patents, Washington, D.C. 20231, on August 29, 2001.

Reinhart, Boerner, Van Deuren, Norris & Rieselbach, s.c.

Dated: August 29, 2001

BY: Rodney D. DeKruif
Rodney D. DeKruif

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Charles F. Struckfus, et al. Attorney Docket No. 4856-CIP

International Application No.: PCT/US00/05364

Serial No.

International Filing Date: March 1, 2000

For: METHOD OF DIAGNOSING AND MONITORING MALIGNANT BREAST
CARCINOMAS

BOX PCT

Assistant Commissioner for Patents

Washington, D.C. 20231

PRELIMINARY AMENDMENT

Sir:

Please amend the attached application to insert this sentence below the title of the invention as a first paragraph of the specification:

This application is a Continuation-in-Part of U. S. Application No. 09/259,993 filed March 1, 1999 and claims priority benefit therefrom.

Remarks

If any clarification is required, the Examiner is invited to telephone the undersigned at (414) 298-8360.

Customer No. 22922

Respectfully submitted,

Reinhart, Boerner, Van Deuren,
Norris & Rieselbach, s.c.
Attn: Linda Gabriel, Docket Clerk
1000 North Water Street, Suite 2100
Milwaukee, WI 53202-0900
414-298-8360

BY: Rodney D. DeKruif
Rodney D. DeKruif
Registration No. 35,853

METHOD OF DIAGNOSING AND MONITORING
MALIGNANT BREAST CARCINOMAS

09/914501

This application is a Continuation-in-Part of U.S. Application No. 09/259,993 filed March 1, 1999 and claims priority benefit therefrom.
Background of the Invention.

This invention relates generally to the use of salivary biomarkers to diagnose breast cancer and, more particularly, to diagnostically differentiate between women with carcinoma of the breast, women with benign tumors, and healthy controls.

Breast cancer is the second leading cause of death among women in the United States. Approximately 1 woman in every 10 will develop breast cancer in her lifetime. Recent statistics estimate that 44,000 women will die of breast cancer, while 150,000 new female cases of breast cancer will be diagnosed in the next year.

It has been shown that screening for breast cancer can reduce breast cancer mortality. Among women aged 50 and older, studies have demonstrated a 20% to 40% reduction in breast cancer mortality for women screened by mammography and clinical breast examination. However, among women between 40 to 49 years of age, the mortality rate is reduced only 13% to 23%. These results suggest that further methods of screening could potentially reduce the mortality in the younger age group of women.

While physical examination and mammography are useful screening procedures for the early detection of breast cancer, they can produce a substantial percentage of false positive and false negative results especially in women with dense parenchymal breast tissue. For example, the probability of having a false negative mammographic examination is 20% to 25% among women between 40 to 49 years of age and 10% among women 50 to 69 years of age. Consequently, screening will result in a number of negative biopsy results yielding a high percentage of false positives. There is also a demonstrated lack of sensitivity in detecting cancerous lesions in younger women yielding a significant percentage of false negatives.

There has also been a clear need for added modalities of screening to help diagnose cancer in younger women. Increased technology in the field of mammography has allowed more reliable detection of small lesions of the breast; while, researchers in the field of breast cancer continue to seek additional adjunct diagnostic procedures to further enhance cancer screening and, thereby, to reduce mortality rates.

09/914501

318 Rec'd PCT/PTO 29 AUG 2001

PATENT

I hereby certify that this paper or fee is being deposited with the United States Postal Service as Express Mail No. EL732810692US in an envelope addressed to: International Preliminary Examining Authority, ATTN: PCT US/RO, Commissioner for Patents, Washington, DC 20231 on April 6, 2001.

Reinhart, Boerner, Van Deuren,
Norris & Rieselbach, s.c.

Dated: April 6, 2001

BY: Rodney D. DeKruif
Rodney D. DeKruif

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of :)
University of Mississippi Medical Center)
Serial No: PCT/US00/05364)
Filed: 01 March 2000)
For: METHOD OF DIAGNOSING AND) Attorney Docket No. :
MONITORING MALIGNANT BREAST) 4856-PCT
CARCINOMAS)

International Preliminary Examining Authority
Commissioner for Patents
Washington, DC 20231

LETTER UNDER RULE 66.8

Dear Sir:

Attached hereto are replacement sheets to account for amendments to several claims, under Article 34(2)(b).

The claims now submitted differ from those originally filed only with respect to claims 1, 10 and 15. The claims are amended to clarify the invention and better distinguish it over the prior art cited in the Written Opinion. The accompanying Response (to the Written Opinion) briefly discusses the prior art, supports the amendments and patentably distinguishes the subject invention over the prior art.

Claims 1, 10 and 15, as originally filed, are hereby amended and provided on replacement sheets 28, 29 and 30, respectively.

If any questions remain as to the claim amendments or the replacement sheets submitted in support thereof, an invitation is hereby extended to contact the undersigned by telephone.

Respectfully submitted,

BY: Rodney D. DeKruif
Rodney D. DeKruif
Attorney for Applicant

Reinhart, Boerner, Van Deuren,
Norris & Rieselbach, s.c.
1000 North Water Street, Ste. 2100
Milwaukee, WI 53202-3186
414-298-8360

Attorney's Docket No. 4856 **PATENT**

Applicant or Patentee: Streckfus, et al.

Serial or Patent No.: 09,259,993

Filed or Issued: March 1, 1999

For: METHOD OF DIAGNOSING AND MONITORING MALIGNANT BREAST CARCINOMAS

**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(c))-NONPROFIT ORGANIZATION**

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF CONCERN University of Mississippi Medical Center

ADDRESS OF CONCERN 2500 North State Street
Jackson MS 39216-4505

TYPE OF ORGANIZATION

- ☒ UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION
- ☐ TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) AND 501(c)(3))
- ☐ NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA
(NAME OF STATE _____)
(CITATION OF STATUTE _____)
- ☐ WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) AND 501(c)(3)) IF LOCATED IN THE UNITED STATES OF AMERICA
- ☐ WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA
(NAME OF STATE _____)
(CITATION OF STATUTE _____)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code with regard to the invention entitled

Method of Diagnosing and Monitoring Malignant Breast Carcinomas

by inventor(s) Charles F. Streckfus, Lenora G. Bigler, James Tate Thigpen

described in

- ☐ the specification filed herewith.
- ☒ application serial no. 09/259,993, filed March 1, 1999.
- ☐ patent no. _____, issued _____.

I hereby declare the rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

MW506327RDD:REG 05/26/99

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights in the invention is listed below* and no right in the invention are held by any person, other than the inventor, who would not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

**NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27).*

NAME _____

ADDRESS _____

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

NAME _____

ADDRESS _____

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small business entity is no longer appropriate. (37 CFR 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING A. Wallace Conerly, M.D.

TITLE IN ORGANIZATION Vice Chancellor for Health Affairs

ADDRESS OF PERSON SIGNING 2500 North State Street, Jackson MS 39216-4505

SIGNATURE 

Date 5/27/99

METHOD OF DIAGNOSING AND MONITORING
MALIGNANT BREAST CARCINOMASBackground of the Invention.

This invention relates generally to the use of salivary biomarkers to diagnose breast cancer and, more particularly, to diagnostically differentiate between women with carcinoma of the breast, women with benign tumors, and healthy controls.

Breast cancer is the second leading cause of death among women in the United States. Approximately 1 woman in every 10 will develop breast cancer in her lifetime. Recent statistics estimate that 44,000 women will die of breast cancer, while 150,000 new female cases of breast cancer will be diagnosed in the next year.

It has been shown that screening for breast cancer can reduce breast cancer mortality. Among women aged 50 and older, studies have demonstrated a 20% to 40% reduction in breast cancer mortality for women screened by mammography and clinical breast examination. However, among women between 40 to 49 years of age, the mortality rate is reduced only 13% to 23%. These results suggest that further methods of screening could potentially reduce the mortality in the younger age group of women.

While physical examination and mammography are useful screening procedures for the early detection of breast cancer, they can produce a substantial percentage of false positive and false negative results especially in women with dense parenchymal breast tissue. For example, the probability of having a false negative mammographic examination is 20% to 25% among women between 40 to 49 years of age and 10% among women 50 to 69 years of age. Consequently, screening will result in a number of negative biopsy results yielding a high percentage of false positives. There is also a demonstrated lack of sensitivity in detecting cancerous lesions in younger women yielding a significant percentage of false negatives.

There has also been a clear need for added modalities of screening to help diagnose cancer in younger women. Increased technology in the field of mammography has allowed more reliable detection of small lesions of the breast; while, researchers in the field of breast cancer continue to seek additional adjunct diagnostic procedures to further enhance cancer screening and, thereby, to reduce mortality rates.

During the past three decades, cancer researchers have made extensive use of immunohistochemistry to detect expression of specific biomarkers that may be used as adjunct diagnostic procedures in the diagnosis of certain tumors. (Grizzle WE. Biomarkers-The New Frontier in the Pathology of Invasive and Preinvasive Neoplasias. Biotechnic and Histochemistry, 72(2):59-61, 1997; Grizzle WE, Myers RB, Manne U. The Use of Biomarker Expression to Characterize Neoplastic Processes. Biotechnic and Histochemistry, 72(2):96-104, 1997.) Tumor markers such as c-erbB-2 (erb) and Cathepsin-D (CD) have been assayed in tissue and shown to correlate with aggressive lesions. The majority of the investigations performed have used these markers in tissues and serum.

With respect to specific cancer antigens in saliva, Chien found that saliva contained CA 125, a glycoprotein complex that is a recognized or accepted tumor marker for epithelial ovarian cancer. (Chien DX, Schwartz PE, CA 125 Assays for Detecting Malignant Ovarian Tumors. Obstetrics and Gynecology, 75(4):701-704, 1990.) In comparing salivary CA 125 concentrations among healthy controls, women with benign lesions, and those with ovarian cancer, Chien found a significantly elevated CA 125 concentration among the ovarian cancer group as compared to the nonmalignant controls. Boyle detected and identified tumor-specific mutations using radio-labeled oligonucleotide in preoperative salivary samples of individuals suffering from head and neck squamous cell carcinoma. These findings were demonstrative in 71% of the patients studied. (Boyle JO, Mao L, Brennan JA, Koch WM, Eisele DW, Saunders JR, Sidransky D. Gene Mutations in Saliva as Molecular Markers for Head and Neck Squamous Cell Carcinomas. Am J Surgery, 168(5):429-32, 1994.)

Summary of the Invention.

However, such antigens are not diagnostic for breast cancer, and the aforementioned tumor biomarkers (*e.g.*, CA 125, erb and CD) have not been tested for their presence in saliva. While the diagnostic methods of the prior art have generally progressed, such innovations have not been extended to all areas of diagnosis. There is a need for a method to more fully utilize recent technological advances and apply them to the detection and treatment of breast carcinomas.

Accordingly, it is an object of the present invention to use saliva as a diagnostic medium and/or as part of a non-invasive protocol for the detection and differential diagnosis of breast carcinomas, thereby overcoming various deficiencies and shortcomings of the prior art, including those outlined above.

It can be another object of the present invention to identify one or more biomarkers present in saliva, as having diagnostic value and/or as can be used in post-treatment monitoring or therapy. Likewise, it can be another object to provide one or more biomarkers as part of a diagnostic panel for the initial detection, follow-up screening for detection, reoccurrence of breast cancer in women, response to chemotherapy and/or surgical treatment of the disease state.

It can also be an object of the present invention to determine one or more appropriate concentration cut-off values for biomarkers diagnostic for the initial detection, follow-up screening for detection, recurrence of breast cancer in women, chemotherapeutic response and/or surgical treatment of the disease state.

It can be another object of the present invention to provide a method of using serum and salivary cut-off concentrations for diagnostic biomarkers to compare detection rates and/or sensitivities. Likewise, it can also be an object of the present invention to provide a method of using receiver operator curves and related analyses to determine cut-off concentrations for a variety of salivary biomarkers having diagnostic value in the detection and/or treatment of breast carcinomas.

It can also be an object of the present invention to use saliva as a medium to determine nodal status of a patient diagnosed with a breast carcinoma. Likewise, it can be a further object of this invention to identify one or more biomarkers present in saliva in the determination of nodal status.

It can also be an object of the present invention to use saliva to determine the receptor status of a biomarker present therein, as part of a differential diagnosis of breast carcinoma. Likewise, the present invention can also include a method of using receptor status of a biomarker present in saliva as an indication of tumor aggressiveness.

It would be understood by those skilled in the art that one or more aspects of this invention can meet certain objectives, while one or more other aspects can meet certain

other objectives. Each objective may not apply equally, in all instances, to every aspect of the present invention. As such, these objectives- -in light of the prior art regarding diagnosis of breast cancer- -can be viewed in the alternative with respect to any one aspect of the present invention.

Other objects, features, benefits and advantages of the present invention will be apparent from this summary and the following inventive examples, and will be readily apparent to those skilled in the art having knowledge of the nature and detection of cancer biomarkers and their use in the diagnosis of corresponding disease states. Such objects, features, benefits and advantages will be apparent from the above as taken in conjunction with the accompanying examples, tables, data and all reasonable inferences to be drawn therefrom.

In part, the present invention is a method of using a salivary biomarker to differentially diagnose and/or detect reoccurrence of breast carcinoma. The method includes (1) using a human saliva specimen to provide a salivary biomarker for that individual and diagnostic for carcinoma of the breast, (2) comparing the individual biomarker with a biomarker reference, and (3) differentially identifying the diagnosis for the individual as indicated by the biomarker comparison. The biomarker reference can be made up of a panel of constituents and can be developed using malignant tumor, benign tumor and control group populations. Each referenced biomarker constituent can have associated with it a range of values comparable to a corresponding individual biomarker.

In preferred embodiments, the individual biomarker is one constituent of a biomarker panel, and the reference panel includes one or more biomarkers identified as having diagnostic value. Such biomarkers can include cancer antigen 15-3, tumor suppressor oncogene protein 53 and oncogene c-erbB-2. In highly preferred embodiments of the inventive method, the presence of oncogene c-erbB-2 and/or an increased expression of protein identifies an individual as having a malignant carcinoma.

Each individual biomarker constituent can be associated with a concentration value, for comparison with a corresponding reference constituent. In one embodiment of the present invention, the concentration of cancer antigen 15-3 for an individual having a

malignant breast tumor is at least about 100 percent higher than such a concentration for an individual having a benign tumor. Likewise, in a preferred methodology, the concentration of oncogene protein 53 for an individual having a malignant breast tumor is at least about 25 percent lower than an individual having a benign tumor. Such differential identifications can be used alone or in conjunction with one or more primary diagnostic methods for the testing and detection of breast carcinomas.

In part, the present invention is a post-operative method of monitoring tumor growth. The method includes (1) providing an individual post-operative to the removal of a malignant tumor, (2) using a saliva specimen from that individual to develop a post-operative biomarker panel, (3) comparing the post-operative biomarker panel with a pre-operative biomarker reference panel for the individual, and (4) determining the presence of malignancy by monitoring at least one constituent of the respective biomarker panels.

Typically, and in preferred embodiments of this method, post-operative chemotherapy is administered to the individual. The chemotherapy can include but is not limited to a therapeutic regimen of cyclophosphamide, methotrexate and fluorouracil. In preferred embodiments, both biomarker panels include a c-erbB-2 constituent, the post-operative detection of which indicates tumor reoccurrence. Alternatively, both biomarker panels can include tumor suppressor oncogene protein 53 as a constituent, the post-operative absence of which indicates tumor inhibition.

In part, the present invention is a method of using the concentration of an endogenously encoded protein to diagnose carcinoma of the breast. The method includes (1) using a saliva specimen from an individual to provide a protein biomarker diagnostic for carcinoma of the breast, (2) comparing the individual protein biomarker with a reference protein, and (3) determining an elevated concentration of the individual protein biomarker over the referenced protein to diagnose the individual. In preferred embodiments, the biomarker protein is one constituent of a biomarker panel. Likewise, the reference protein can be one constituent of a reference panel. Regardless, any such protein can be developed as a reference using malignant tumor, benign tumor and control

group populations. In highly preferred embodiments, the individual protein biomarker is cancer antigen 15-3 or, alternatively, an expression of oncogene c-erbB-2.

The biomarkers of the present invention can include any proteinaceous expression, fragment or bioderivative, or ligand or antibody thereto, encoded by any oncogenetic material, which has or can be characterized biochemically, physiologically or structurally.

For instance, CA15-3 has been characterized as a mucinous glycoprotein and shown to be a diagnostic indicator. More specifically, CA15-3 is a carcinoma-associated antigen which is identified by two monoclonal antibodies designated Mab D11-5 and Mab DF3. Mab D11-5 is prepared against an antigen of human milk fat globule membranes, and Mab DF3 is generated against membrane fraction from human breast cancer.

It has also been observed that the c-erbB-2 oncogene (also referred to as HER-2/neu), which is capable of transforming cells to malignancy, is present in some tumors at very high levels [Zhou et al., Cancer Research, 47:6123 (1987); Berger et al., Cancer Research, 48:1238 (1988); Kraus et al., The EMBO Journal, 6 (3):605 (1987); and Slamon et al., Science, 235:177 (1987)]. The expression of the c-erbB-2 oncogene, and its location in the external membrane of cells appears to be closely associated with cancer [Kraus et al., id.; Slamon et al., id.; Drebin et al., Cell, 41:695 (1985); and Di Fiore et al., Science, 237:178 (1987)]; it may, in fact, be the primary event in the development of cancer in at least some cases [Muller et al., Cell, 54:105 (1988)]. Overexpression of the c-erbB-2 protein on the surface of normal cells appears to cause them to be transformed or otherwise behave as tumor cells.

Evidence of such transformation can be found, of course, proximate to the disease. Underlying many facets of diagnostic utility, however, is the discovery that c-erbB-2 overexpressing cells shed the c-erbB-2 external domain into neighboring tissues. Derivatives of c-erbB-2 have been found in the serum of stably transformed expressing cells.

A glycoprotein having an approximate molecular weight of 75 kilodaltons (kd) has been identified to constitute the external domain of the approximately 185 kd glycoprotein (gp185) that is c-erbB-2. The term "gp75" is precisely defined by its

nucleotide and amino acid sequences; the gp75 external domain comprises the region from about amino acid number 22 (serine: ser-22) to about amino acid number 653 (serine; ser-653) with the nucleotide sequence corresponding thereto. The amino acid sequence represents the nonglycosylated version of gp75 which would be expected to have a molecular weight corresponding thereto.

The gp75 proteins and polypeptides are encoded by the gp75 external domain DNA sequence (nucleotides encoding from approximately ser-22 to approximately ser-653) or by fragments of said gp75 DNA sequence. The phrase "gp75 proteins and polypeptides" is therefore interpreted to include proteins and/or polypeptides that have substantially the same amino acid sequences or portions thereof, and/or substantially the same biological activity as the gp75 proteins and polypeptides.

The present invention shows such proteinaceous materials, once found elsewhere, can also be identified and further characterized in saliva. For example and as discussed elsewhere herein, it was undertaken to determine if the oncogene, HER-2/neu, was present in Stimulated Whole (SWS), Parotid (P2), submandibular/sublingual (S2) and/or Minor (M2) salivary secretions among six healthy, age matched women. Because of its relationship with saliva, gingival crevicular fluid (GCF) was also assessed. HER-2/neu assays were performed by ELISA. HER-2/neu concentrations were assayed in serum and compared to those of saliva. Assays revealed the presence of HER-2/neu in SWS (40.71 Units/ml), PS (15.71 Units/ml), and S2 (14.08 Units/ml) with only trace amounts appearing in M2 and GCF. SWS produced the highest levels of HER-2/neu as compared to glandular secretions. Overall and as might be expected, the greatest concentration of HER-2/neu appeared in serum. However, when HER-2/neu concentrations were corrected for total protein, the higher concentrations appeared in P2 (72.78 Units/ml) secretions, with lesser amounts excreted in SWS (34.01 Units/ml) and S2 (34.95 Units/ml) by comparison.

Such results indicate that the protein, HER-2/neu, is present in saliva and is conveyed, primarily, by the parotid gland. The results also indicate that HER-2/neu may passively diffuse from the serum, to the interstitium, and then be excreted by saliva into the oral cavity. A growing body of work relates to saliva constituents and mechanisms of salivary secretion (See Glandular Mechanisms of Salivary Secretion, Edited by Garrett et

al., (1999) and the references cited therein), demonstrating skill in the art correlating serum and salivary proteins.

In accordance with the present invention, proteins -- in addition to the c-erbB-2 proteins described herein -- distinctly associated with either breast cancer or other carcinogenic disease states have been or can be analyzed or characterized in a manner similar to that described above for the c-erbB-2 oncogene. As would be known to those skilled in the art and subsequently made aware of this invention, those other distinctly disease associated proteinaceous expressions, whether identified proximate to the disease or found serially, are detectable in saliva and can be evaluated as described herein for use as salivary biomarkers diagnostic for associated disease states.

The biomarkers and related inventive method can be used for detecting breast carcinoma and provide for an economical and logistical adjunct diagnostic test for mammography. Furthermore, these salivary markers can also, in conjunction with physician and self breast examination, help to reduce morbidity and mortality rates for breast cancer and thereby reduce overall national health care expenditures.

Brief Description of the Drawings.

Figure 1 is a compilation of mean values for healthy controls, individuals having benign lesions and carcinomas in situ (Ca in Situ), comparing saliva and serum media: ‡ = erb control (saliva) < erb cancer group (saliva) one way sample test t-test (mean vs constant): t-value = 14.31, $p > 0.0001$; ‡‡ = erb control (serum) < erb cancer group (serum) one way sample t-test (mean vs constant) t-value = 10.33, $p < 0.0001$; # = CA 15-3 control & benign (saliva) < CA 15-3 cancer group (saliva) Anova $p < 0.05$; and # # = CA 15-3 control & benign (serum) < CA 15-3 cancer group (serum) Anova $p < 0.01$.

FIGURE 2 is a tabular comparison of salivary and serum concentrations (U/mg protein) of CA 15-3, by diagnostic status.

FIGURE 3 is a tabular comparison of salivary and serum concentrations (U/mg protein) of erb, by diagnostic status.

FIGURE 4 is a tabular comparison of salivary and serum concentrations (fmol/mg protein) of EGFR, by diagnostic status.

FIGURE 5 is a tabular comparison of salivary and serum total protein concentrations (mg/ml), by diagnostic status.

FIGURE 6 is a tabular comparison of salivary and serum concentrations (pmol/mg protein) of p53, by diagnostic status.

FIGURE 7 is a tabular comparison of salivary and serum concentrations (pmol/mg protein) of CD, by diagnostic status.

FIGURE 8 presents mean and standard error values determined for various serum and salivary erb characteristics.

FIGURE 9 presents questionnaire data obtained from the indicated groups studied, showing the utility of the present invention.

FIGURE 10 shows erb values determined by malignant tumor stage.

FIGURE 11 shows a series of cut-off values for erb and CA 15-3 concentrations, in accordance with the various diagnostic methods of this invention.

FIGURE 12a shows graphically mean values, 95% Confidence Intervals, and cut-off value (110 Units/ml) for salivary c-erbB-2 Units/ml for the control group, group diagnosed with benign lesions, and the group diagnosed with carcinoma of the breast.

FIGURE 12b shows graphically, mean values, 95% Confidence Intervals, and cut-off value (110 Units/ml) for salivary c-erbB-2 Units/mg of protein for the control group, group diagnosed with benign lesions, and the group diagnosed with carcinoma of the breast.

FIGURE 13a shows, a graphically, mean values, 95% Confidence Intervals, and cut-off value (2000 Units/ml) for serum c-erbB-2 Units/ml for the control group, group diagnosed with benign lesions, and the group diagnosed with carcinoma of the breast.

FIGURE 13b shows graphically mean values, 95% Confidence Intervals, and cut-off value (50 Units/ml) for serum c-erbB-2 Units/mg of protein for the control group, group diagnosed with benign lesions, and the group diagnosed with carcinoma of the breast.

FIGURE 14a shows graphically mean values, 95% Confidence Intervals, and cut-off value (4.0 Units/ml) for salivary CA 15-3 Units/ml for the control group, group diagnosed with benign lesions, and the group diagnosed with carcinoma of the breast.

FIGURE 14b shows graphically mean values, 95% Confidence Intervals, and cut-off value (20 Units/ml) for serum CA 15-3 Units/ml for the control group, group diagnosed with benign lesions, and the group diagnosed with carcinoma of the breast.

FIGURE 15 shows a graphic plot of receiver operating characteristic (ROC) curves (sensitivity vs. 1-sensitivity) for salivary c-erbB-2 Units/ml (—), salivary c-erbB-2 Units/mg of protein (····), and salivary CA 15-3 Units/ml (---); the percent area under each curve is as follows: c-erbB-2 ~76%, a cut-off of about 110 units/ml; c-erbB-2/tp ~77%, a cut-off of about 100 Units/mg protein; and CA 15-3 ~71%, a cut-off of about 4 Units/ml.

FIGURE 16 shows graphically receiver operating characteristic (ROC) curves (sensitivity vs 1-sensitivity) for serum c-erbB-2 Units/ml (—), serum c-erbB-2 Units/mg of protein (····), and serum CA 15-3 Units/ml (---); percent area under each curve is as follows: c-erbB-2 ~77%, a cut-off of about 2000 Units/ml; c-erbB-2/tp ~76%, a cut-off of about 50 Units/mg protein; and CA 15-3 ~71%, a cut-off of about 20 Units/ml.

Examples of the Invention.

The following non-limiting examples and data illustrate various aspects and features relating to the method(s) of the present invention, including the surprising and unexpected results obtained thereby.

With respect to the following examples and data, the subject population consisted of 21 women from the general population (controls) and the University of Mississippi Medical Center (UMMC), Department of Oncology and Surgery Clinics (tumor patients). Individuals with a breast mass were referred to UMMC from the surrounding community for evaluation. Each patient was given a thorough physical examination and evaluated for carcinoma of the breast. Saliva and serum specimens were collected from each women at the initial visit at the clinic and prior to receiving any treatment. Final pathologic diagnostic evaluations later revealed whether the individual had a benign tumor, or carcinoma of the breast (in situ). Investigators were initially blind with respect to diagnostic outcome of the subjects until a final diagnosis was rendered by the pathologist and the patient referred for further treatment. The subjects were racially mixed and ranged in age from 30 to 80 years.

False positive results were eliminated. It was initially envisioned that the present methodology might provide false positives due to extraneous physiological and environmental factors such as estrogen levels and smoking, respectively. However, such factors have been eliminated as providing false positive results. Race, age, menopausal status, medication usage and health status were also eliminated as factors producing false positive results.

Assays were determined as indicated using the referenced commercial kits and associated reagents, procedures and/or techniques. Kits from Triton Diagnostics are no longer available. Kits from CIS bio international are particularly useful and provide enhanced sensitivity, especially with regard to the erb marker.

Example 1

Statistical Analysis. Statistical analysis were performed using the SPSS statistical software package. A descriptive analysis was made comparing mean marker values for the controls, those with benign tumors, and carcinoma of the breast.

A one-way analysis of variance for unbalanced data, the general linear models procedure, was used to compare the mean values for the group with breast carcinoma with a non cancer groups. The polynomials formulated using the general linear models procedure are easy to interpret and are appropriate for all sample sizes including those too small to sustain an appropriate multivariate analysis. The Tukey post-hoc analysis was used for significant linear models.

Considering that erb was undetected among controls and benign lesions for both saliva and serum, a one way sample t-test was performed. Due to the small sample size, issues concerning the specificity and sensitivity of the panel of markers were not addressed, but will be investigated in subsequent studies.

Example 2

Specimen Collection. Stimulated whole saliva specimens were collected for a 5 minute period using a cube of paraffin as a stimulant (Navasesh, 1982)¹⁷ Salivary flow rates were determined gravimetrically. All specimens were collected in the morning thereby controlling for any possible effects that circadian rhythm may produce in marker concentration. Samples can be frozen for future analysis. Blood was also drawn at the

time of saliva collection by a phlebotomist. None of the participants exhibited cancerous or precancerous lesions in the oral cavity at the time the specimens were collected.

The frozen saliva samples were thawed and centrifuged at 500-1500G for 20 min to precipitate cells and mucin in order to extract the bio-marker proteins. The clear saliva extract and the serum from the blood specimens were analyzed for total protein and the panel of biomarkers.

Example 3

Total Protein. A colorimetric assay for measuring total protein concentration, based on the color change of Coomassie brilliant blue G-250 dye in response to various concentrations of proteins, was used (Bio-Rad Kit). Specimens were read on a spectrophotometer and absorbance measured at 595nm. Total protein concentration of the samples was determined from a standard curve constructed with bovine gamma globulin standards.

Example 4

CA 15-3. CA 15-3 assays were determined by using EIA kits (CIS bio international). The CA 15-3 assay is a two-site solid phase enzyme immunoassay. The molecules of CA 15-3 are "sandwiched" between two monoclonal antibodies, the first one attached to the ELSA solid phase and the second one linked to the horseradish peroxidase (enzymatic conjugate). After washing, the enzymatic reaction develops a color proportional to the amount of CA 15-3 present in the assay. Absorbances are read at 490 nm using a spectrophotometer and concentrations are calculated from standard curves constructed from known concentrations of the ligand. The CA 15-3 assay is designed to assay serum specimens. Saliva supernatants were substituted in place of the serum for salivary CA 15-3 determinations. The antibodies used in the test do not present cross-reaction with other known tumor markers (CEA, CA 19-9, CA 125) and the salivary concentrations are substantially above the lower limit of detection for the assay. CA 15-3 concentrations were expressed as units/mg of protein.

Example 5

erb and pantropic p53. erb and pantropic p53 assays were determined using ELISA kits (Oncogene Research, Co.). In this study serum and the salivary supernatant

were substituted in place of the tissue extracts as assay specimens. A colorimetric evaluation of the level of binding was performed and the intensity of the color formed by the enzymatic reaction is proportional to the target protein present. Absorbances were read at 490 nm in a microplate spectrophotometer and ligand concentrations calculated from standard curves. erb and p53 data were expressed as units/mg of protein and pmol/mg of protein, respectively. The antibodies used in the test do not present cross-reaction with other known tumor markers and the salivary concentrations are substantially above the lower limit of detection for the assay.

Example 6

Cathepsin-D Assay. Salivary and serum CD concentrations were determined using enzyme immunoassay (EIA) kit (Triton Diagnostics, Inc.). A monoclonal antibody and a rabbit polyclonal antibody both specific for CD were simultaneously incubated with both the saliva and serum specimens. During the incubation, the CD present in the saliva and serum specimens was bound by the two anti-CD antibodies. The monoclonal antibody is conjugated to biotin causing the formed antigen-antibody complex to be bound onto the streptavidin-coated tube. Unbound materials were removed by washing the tubes. In the second incubation, an anti-rabbit antibody conjugated with horseradish peroxidase was added to the tube. The conjugate was then bound to the complex. Unbound complex was removed by a second washing. The tubes were then incubated with a TMB substrate solution in order to develop a color. Phosphoric acid was then added to stop the enzymatic reaction. The intensity of the color that was developed was determined using a spectrophotometer set at 450 nm. Specimen values were determined from the curve which resulted by plotting the absorbance values of the controls against the known concentrations (pmol/mg of protein).

Example 7

Epidermal Growth Factor Receptor. EGFR assays were determined using EIA kits (Triton Diagnostics, Inc.). The anti-EGFR conjugate was incubated with the saliva and serum specimens. During the incubation the EGFR protein becomes bound by the anti-EGFR conjugate. One of the monoclonal antibodies is conjugated to horse radish peroxidase. During the second incubation the resulting immune complexes become

bound onto a coated polystyrene tube by a "linking solution". Unbound substrates were then washed by decanting. The tubes were then incubated with a TMB substrate solution in order to develop a color. Phosphoric acid was then added to stop the enzymatic reaction. The intensity of the color that was developed was determined using a spectrophotometer set at 450nm. Specimen values were determined from the curve which resulted by plotting the absorbance values of the controls against the known concentrations (fmol/mg of protein).

For all their power, immunoassays are subject to many kinds of interference. The investigators performed several test laboratory tests to control for these problems. With respect to ligand recovery, the investigators were able to establish the amount of marker (ligand) recovered from saliva and serum samples. Five saliva and serum specimens with known amounts of marker were serially diluted. The dilutions were assayed for all three markers. The data were plotted against the expected values to determine the linearity of dilution. The slopes of both the dose response curve and the standard curve were not significantly different from each other and the intercepts were not significantly different from zero. During the assaying of the specimens, the investigators employed the use of appropriate positive and negative controls for all marker assays. When performing the assays, some test specimens contained primary antibodies preincubated with excess ligand to control for false positives. In addition, test specimens were preincubated with excess free primary antibody to determine if the signal had been eliminated. These extra tests provided additional quality control during the course of specimen analyses. When assayed, all specimens were run in triplicate.

The control group consisted of 15 women (age 42.4), the benign tumor group consisted of 8 women (age 45.3), and the cancer group consisted of 12 women (age 49.0). The subjects diagnosed with benign lesions consisted of women with fibroadenomas (n=4), lipomas (n=1), and fibromas (n=3). The women with breast cancer were diagnosed with lobular carcinoma (n=1), infiltrating ductal carcinoma (n=9), and ductal carcinoma *in situ* (n=2). All of the subjects with carcinoma of the breast were node negative and without evidence of metastases. Five of the cancer subjects among the cancer group were edentulous while only two among the non-cancer group were

edentulous. All other subjects were dentate. The mean values for the three groups are shown in FIG. 1 and illustrated graphically in FIGS. 2-7.

As shown in FIGS. 1 and 2, the mean values for CA 15-3 among the controls and benign lesions group were approximately 45%-50% lower than the mean value for the cancer group. This was statistically significant at the $p < 0.05$ level for saliva and $p < 0.01$ level for serum.

Referring to FIGS. 1 and 3, erb was not detected in the saliva or the serum of the controls or benign lesions group. Conversely, the carcinoma group exhibited the presence of erb and the t-test showed significantly higher concentrations ($p < 0.001$).

Additionally, p53 levels were approximately 25% higher among the controls and the benign lesion groups as compared to the cancer group (FIGS. 1 and 6.) The investigators expected higher p53 values among the controls as compared to those women with breast cancer in so far as p53 mutation reflects the inability of the oncogene to render tumor suppression. As shown in the accompanying figures, saliva and serum levels of CD and EGFR did not appear to be as tumor specific as CA 153, erb and p53 when compared across the three groups of women.

Example 8a

With respect to the presence of the panel of markers in saliva, several technical issues were also addressed. One such issue was to determine if cells from the oral epithelium may possibly contribute to marker levels found in the saliva. To address this, salivary specimens were centrifuged and the supernatant separated from the pellet. A sample from the supernatant was placed on a glass slide, stained and microscopically examined for the presence of cells. The examination disclosed the absence of cells in the supernatant. Next, the pellet was resuspended in phosphate buffered saline. Both the supernatant and the resuspended pellet were analyzed for the presence of the biomarkers. The results showed biomarker levels in the supernatant, but an absence of biomarkers in the resuspended pellet, indicating the biomarkers originate in the saliva and that there are no biomarker contributions from the cells.

Example 8b

A second experiment was performed using secretory IgA (slgA) as a control protein to compare individuals with and without carcinoma of the breast. The predominant immunoglobulin in saliva is slgA. It is derived from salivary glands with the parotid gland being the principal producing gland. The antibody is synthesized as IgA dimers by immunocytes present in the major and minor salivary glands. Because of its ability to attenuate pathogenic assault, slgA is considered to be the oral cavity's first line of defense. This salivary protein has no relationship with carcinoma of the breast and was selected as a control protein. Using ELISA methodology, slgAs were detected in the saliva from both the cancer and the non-cancer groups. The results of this test showed no significant differences among those individuals with (\bar{x} 11.7 ng/ml) and without cancer (\bar{x} 14.3 ng/ml), indicating that the only proteins which appear to be elevated are those markers associated with carcinoma of the breast.

Example 8c

A third experiment was performed to determine the effects of oral health on the marker levels. A small number of individuals with periodontal disease was compared to healthy controls and several edentulous subjects. The results showed no significant difference in marker levels among those with periodontal disease, those who were orally healthy and those who were edentulous.

Example 8d

A fourth experiment was conducted to determine the effects of the estrous cycle on salivary marker levels. Two healthy women with regular menses had saliva specimens collected daily from the beginning of their menstrual cycle to its end. The results showed no major fluctuations of salivary marker concentrations occurring during the menstrual cycle. Marker concentrations were relatively consistent over the 30 day period suggesting minimal individual variability (data not shown).

Example 8e

Another experiment was conducted to determine the origin of the salivary gland constituents. Parotid, submandibular, sublingual and minor gland secretions were collected. The results of this experiment indicate that these markers are primarily

secreted by the parotid gland. Parotid gland secretions were found to be many times higher than the submandibular, and sublingual concentrations. Minor gland contributions were barely detectable. Additionally, marker concentrations appear to be flow rate independent.

As shown above, detectable levels of the breast tumor markers CA 15-3, erb, EGFR, CD, and p53 were present in the saliva and serum of women with malignant breast lesions. These markers are also detectable in the saliva and serum of women with benign breast lesions and completely healthy individuals. The results also indicate lower levels of CA 15-3, erb, in noncancer individuals as compared to those with breast carcinoma (Figures 1). The reverse was true with respect to p53.

Several potential confounding factors were also considered and resolved. Accordingly, it was determined that: 1) the cells from the oral epithelium did not contribute to the marker levels, 2) using sIgA as a control protein, the only proteins which are elevated are those markers associated with carcinoma of the breast, 3) the presence of periodontal disease has no effect on marker levels, 4) the estrous cycle had no effect on salivary marker levels, 5) the markers are secreted primarily from the parotid gland and 6) are flow rate independent.

A similar study consisted of three groups of women: Group I was a control group. This group consisted of healthy, asymptomatic individuals from the University of Mississippi Medical Center (UMMC). Health status for the control group was determined by questionnaire.

Group II, the benign tumor group, and Group III, the malignant tumor group, consisted of consecutive individuals from the surrounding community with a breast mass that were referred by a physician to UMMC Division of Oncology for evaluation. Each patient received a thorough physical examination and was evaluated for carcinoma of the breast. Saliva and serum specimens were collected from each woman at the initial clinic appointment and prior to receiving any treatment. Final diagnostic evaluations obtained from pathology reports determined whether the individual would be classified to Group II, the benign tumor group, or to Group III, the group diagnosed with carcinoma of the

breast. Staging and nodal status were assessed according to the criteria set forth by the American Joint Committee on Cancer.

All participants were administered a brief questionnaire at the time of signing the IRB approved consent form. This data was collected by interview and included information concerning their age, race, tobacco usage, pharmacological and medical histories, and menopausal status.

Similar to the procedures and/or examples discussed above, stimulated whole saliva specimens were collected for a 5 minute period using a cube of gum base as a stimulant following standardized collection procedures. Upon collection, the specimens were aliquoted and frozen for analysis. Salivary flow rates were determined gravimetrically. All specimens were collected in the morning thereby controlling for any possible effects that circadian rhythm may produce in marker concentration. Blood was also drawn at the time of saliva collection by a phlebotomist.

The frozen specimens were thawed and the saliva and the serum from the blood specimens were analyzed for total protein and the *c-erbB-2* concentrations.

The specimens were also assayed for CA 15-3. The effectiveness of CA 15-3 as a diagnostic marker is documented in the literature and was used as a reference marker or "diagnostic gold standard" by which to compare the efficacy of the *c-erbB-2* marker.

Samples of saliva were assayed for protein using the bicinchoninic acid method (Pierce Chemical, Co.) which is a highly sensitive and selective detection reagent for the cuprous ion. This method measures protein concentrations from 0.5-20 mg/ml. In this assay, bicinchoninic acid serves as a chelating agent for Cu^{+1} forming a color complex in the presence of protein. Aliquots of saliva (100 μL) were placed in microtiter plates and the Pierce BCS protein assay reagent added to the wells. Samples were incubated for 30 minutes at 37°C and the optical density read at 562 nm in a microplate spectrophotometer. The final concentration of each substance was derived from a standard curve and data was expressed as mg/mL.

Serum and salivary extracellular domain *c-erbB-2* antigen levels were assayed using ELISA kits from Oncogene Research Products. Whole saliva was substituted in place of serum as assay specimens. The basic assay involves a colorimetric evaluation of

the level of binding which was performed and the intensity of the color formed by the enzymatic reaction being proportional to the target protein present. The absorbance was read at 490 nm in a microplate spectrophotometer and the ligand concentration calculated from a standard curve. *c-erbB-2* data were expressed and reported as both Units/ml and Units/mg of protein so that these findings could be compared to previous results in the literature.

CA 15-3 assays were determined by using ELA kits from CIS Bio International. The CA 15-3 assay is a two-site solid phase enzyme immunoassay. The molecules of CA 15-3 are "sandwiched" between two monoclonal antibodies. The first one is attached to the ELISA solid phase and the second one linked to the horseradish peroxidase (enzymatic conjugate). After washing, the enzymatic reaction develops a color proportional to the amount of CA 15-3 present in the assay. Absorbance is read at 490 nm (horseradish peroxidase) using a spectrophotometer and the concentration is calculated from a standard curve constructed from known concentrations of the ligand. The CA 15-3 assay is designed to assay serum specimens. Whole saliva was substituted in place of the serum for salivary CA 15-3 determinations. CA 15-3 concentrations were expressed as Units/ml.

Statistical analyses were again performed using the SPSS™ statistical software package. These data were analyzed from four different perspectives. Initially, the saliva and serum marker concentrations were summarized for each group and descriptive analyses were conducted for the demographic and supplemental data obtained from the questionnaire. The focus was on race, medical status, tobacco use, medication usage, and menopausal status with respect to *c-erbB-2* concentrations. The data were summarized by tumor type, staging, and nodal status. Due to the small number of women in the cancer group, the number of sub-categories for primary tumor (T) and nodal status (N) were collapsed. The primary tumor categories were dichotomized to T1 and greater than T1, while nodal status was reduced to node negative and node positive, respectively.

A one-way analysis of variance (ANOVA) was used to compare the mean marker values for the three groups, focusing on the breast cancer in contrast to the non-cancer groups. The Dunnett's test was used to adjust for multiple comparisons.

Possible associations among the salivary and serum *c-erbB-2* levels as well as those between *c-erbB-2* and CA 15-3 concentrations in saliva and serum were investigated by Pearson's correlation coefficient. As the distribution of some of these concentrations were skewed, the data were transformed using the square root of each value.

Example 9

Receiver operating characteristic (ROC) analyses were conducted to investigate the appropriate cut-off values for each biomarker. Separately for each marker their concentrations were recoded into dichotomous variables using the mean of the control and the cancer group as the initial cut-off value. Incremental ranges of cut-off values in a positive ($> \bar{x}$) and negative ($< \bar{x}$) direction about the mean were assessed for each marker. Breast cancer was dichotomized into positive and negative. Two-by-two tables were used to compute the sensitivity and specificity values of each biomarker for detecting disease for each cut-off value. ROC curves (sensitivity vs 1-specificity) were constructed for *c-erbB-2* and CA 15-3 concentrations in both saliva and serum. The optimum cut-off value for each marker was determined by using the cut-off value that produced the largest percentage of area under its ROC curve. See, Wilcosky TC. Chapter 3, Criteria for selecting and evaluating markers. In: Hulka BS, Wilcosky TC, Griffith JD, eds: *Biological Markers in Epidemiology*, New York. Oxford University Press, 1990, pp. 36-42; and SPSS for Windows, release 9.0. Chicago: SPSS, 1999.

Example 10

Demographic and supplemental data obtained from the questionnaire were conducted for the three groups of women are summarized in Figures 8 and 9. Frequency comparisons by race, tobacco use, medication use and menopausal status were conducted. There were significant differences in race, tobacco use, and menopausal status among the three groups. More African-Americans experienced carcinoma of the breast and benign tumor lesions than Caucasians. Likewise, significantly more tobacco users experienced carcinoma of the breast and benign tumor lesions than non-users. With respect to menopausal status, perimenopausal women experienced carcinoma of the breast and benign tumor lesions than the pre and postmenopausal women. Mean *c-erbB-2* values

were compared for each group according to health status (e.g., control, benign, cancer). There were no significant reportable effects on *c-erbB-2* concentration attributable to these variables within each group of women (health status). Age comparisons yielded no significant group differences for *c-erbB-2* values and was not linearly related to *c-erbB-2* values when regression modeling was employed.

Further analyses showed that the women with breast cancer produced detectable salivary levels of *c-erbB-2* that were significantly higher than those produced by the benign tumor and the control groups. The means, standard error of the means, and the 95% confidence intervals for the salivary marker concentrations across the three groups are shown in Figures 8, 12-14. As shown in Figure 8, the mean *c-erbB-2* values for the control and benign tumor groups were approximately 50%-57% lower than the mean value for the cancer group. A strong parallel response in the corresponding serum *c-erbB-2* levels was evidenced in these women, with an associated range of 55% to 64%, although the concentrations in serum were roughly 15 times higher than those in saliva before correcting for total protein.

The majority of the benign tumors were fibroadenoma or fibrocystic tumors. There was little difference between salivary *c-erbB-2* concentrations found in fibroadenoma and fibrocystic tumors among women who had these benign tumors. Two women presented with fluid filled cysts and two with benign calcifications. Both the serum and saliva *c-erbB-2* values for the fluid filled cysts were statistically lower than those for the fibroadenoma and fibrocystic groups. Again the responses in serum were similar to those in saliva for these two groups of benign tumors, although the rank ordering of the observed average concentrations reversed between the fibroadenoma and fibrocystic groups.

The vast majority of tumors in the cancer group were infiltrating ductal carcinomas (n=19). One woman had an infiltrating lobular carcinoma, three had a ductal carcinoma, and seven had miscellaneous breast malignancies. The mean salivary and serum *c-erbB-2* concentrations for these groups were all substantially higher than those observed for the benign tumors.

With respect to the staging of the cancer tumors, there was one Stage 0 (T0N0M0) patient, six were Stage I (T1N0M0), eight Stage IIA (T2N0M0), three Stage IIB. The IIB group was composed of one T2N1M0 and two T3N0M0. There were two Stage IIIA composed of one T3N0M0 and one T3N2M0, three Stage IIIB composed of one T3N3M0 and two T4N1M0. Seven patients were not staged at the time the data of this example was made available.

Seven subjects with carcinoma of the breast were node positive and 16 were node negative. All individuals diagnosed with cancer were without evidence of distant metastases. The sub-categories for primary tumor were collapsed into T1 and greater than T1 and node positive and node negative groups (Figure 10). These analyses showed no differences with respect to tumor size for *c-erbB-2* saliva and serum concentrations, but there was an elevated *c-erbB-2* concentration difference between node positive and node negative individuals regardless of diagnostic medium (Figure 10).

Example 11

The second level of analyses compared group means for the women with carcinoma of the breast, women with benign lesions, and the healthy control group. A one-way ANOVA for unbalanced data was performed across the three categories of women for salivary *c-erbB-2* and was found to be significant at the $F=13.83$; $p<0.0001$ level. The Dunnett's C post-hoc analysis exhibited a significant difference between the cancer group and the benign tumor and control groups at the $p<0.001$ level.

A similar result was demonstrated for serum *c-erbB-2* across the three groups of women. The overall ANOVA was significant at the $F=19.95$; $p<0.0001$ level with the post-hoc analyses significant at the $p<0.001$ level (cancer > both non-cancer groups).

With respect to CA 15-3, the overall ANOVA was significant at the $F=5.94$; $p<0.04$ level with the post-hoc analyses significant at the $p<0.05$ level (cancer > non-healthy control group). Similarly, the results for serum *c-erbB-2* across the three groups of women were significant at the $F=20.96$; $p<0.0001$ level with the post-hoc analyses significant at the $p<0.001$ level (cancer > healthy control group).

Data for the salivary and serum *c-erbB-2* levels corrected for total protein concentrations exhibited the same results as the non-corrected data. The overall ANOVA

for salivary *c-erbB-2* was significant at the $F=13.80$; $p<0.0001$ level with the post-hoc analyses significant at the $p<0.001$ level (cancer > both non-cancer groups). The results for serum *c-erbB-2* across the three groups of women were significant at the $F=14.45$; $p<0.0001$ level with the post-hoc analyses significant at the $p<0.001$ level (cancer > both non-cancer groups).

Example 12

The third level of analyses, correlations coefficients, revealed a significant moderate association between serum and salivary *c-erbB-2* at the $r=0.51$; $p<0.0001$ level. There was a significant, moderate association between serum *c-erbB-2* and serum CA 15-3 concentrations at the $r=0.40$; $p<0.001$ level. With respect to serum *c-erbB-2* concentrations corrected by total protein and their association with CA 15-3, the results exhibited a significant, moderate association $r=0.36$; $p<0.001$ level. A slight relationship was found between salivary *c-erbB-2* concentrations corrected by total protein and serum *c-erbB-2* concentrations corrected by total protein at the $r=0.39$; $p<0.001$ level.

Example 13

The comparison of receiver operator curves (fourth level analyses) suggested a cut-off value of 110 Units/ml for salivary and 2000 Units/ml for serum *c-erbB-2* concentrations (Figures 11, 12a and 13a). A comparison of receiver operating characteristic curves was also performed on salivary and serum *c-erbB-2* concentrations corrected by total protein. These values were 100 Units/ml and 50 Units/ml for salivary and serum *c-erbB-2* concentrations (Figures 11 and 12b and 13b). Salivary CA 15-3 determinations yielded a 4.0 Units/ml cut-off value (Figures 11 and 14a). The cut-off value for serum CA 15-3 was 20 Units/ml (Figures 11 and 14b), compared to literature values ranging from 15-40 Units/ml.

Using the aforementioned cut-off values, salivary and serum *c-erbB-2* concentrations were able to detect 87% and 94% of the subjects with cancer, respectively (Figure 11). The salivary and serum *c-erbB-2* concentrations corrected by total protein detected 77% and 84% of the subjects. This compares to 62% and 75% for the salivary and serum CA 15-3 marker. CA 15-3 levels were able to detect 65% of the malignant lesions (Figures 11, 15 and 16).

Example 14

Prior studies involving the *c-erbB-2* oncoprotein vary in the types of populations studied with respect to staging, tumor type, nodal involvement, and the presence of metastases. Additionally, a variety of analytical techniques have been used to study the *c-erbB-2* oncoprotein in both tissue and serum. With respect to serum, most studies have used enzyme-based immunoassays. These techniques have varied with respect to the sensitivity of the assays and the use of either monoclonal or polyclonal antibodies. Some kits used in the literature have since been discontinued and are no longer available to researchers. The findings herein are compared with studies using similar, sample sizes, staging, and assay technology.

The results of the preceding examples suggest elevated salivary *c-erbB-2* and serum *c-erbB-2* levels among women with carcinoma of the breast (Figures 8 and 12-14). With respect to elevated serum *c-erbB-2* levels among breast cancer patients, the findings of this study agree with others found in the literature particularly those evaluating non-metastatic cancer. There is only one report in the literature concerning elevated salivary *c-erbB-2* concentrations among women with breast cancer and that was a preliminary study performed by the authors of this investigation. This earlier study used EIA (Triton, Co.) assay for determining salivary and serum *c-erbB-2* concentrations. The results of that study also revealed significantly higher salivary *c-erbB-2* concentrations among women with carcinoma of the breast. The assay employed in this study, when compared to the assay of the first study using the same identical specimens, appears to be five times more sensitive than the original assay.

Example 15

Benign and malignant tumor comparisons yielded potentially useful information. Subjects with fibroadenomas and fibrocystic lesions produced similar salivary and serum *c-erbB-2* concentrations (Figure 9). For serum, this finding compares with results obtained by Breuer (1998). Subjects diagnosed with infiltrating ductal carcinoma dominated the cancer population in this study. Consequently, comparisons among the various types of malignant breast lesions were not made.

Example 16

Further analyses of the primary tumor data revealed no substantial salivary or serum *c-erbB-2* concentration differences for groups T1 and greater than T1 (Figure 10). This observation does agree with the findings of Watanabe (1994) and Kynast (1993). The finding also suggests that *c-erbB-2* receptor status may be more indicative of tumor aggressiveness than tumor volume.

Example 17

With respect to nodal status, node positive patients *c-erbB-2* levels were found to be elevated when compared to the node negative subjects (Figure 9).

Example 18

The data shows an association ($r=0.51$; $p>0.0001$) between soluble salivary concentrations of *c-erbB-2* and serum levels of *c-erbB-2*. The unexplained variability may be attributed to the "pooling" of the various types of individuals across the three groups of women and the fact the investigators do not discern the exact mechanism by which the *c-erbB-2* protein migrates from the tumor site and enters the oral cavity (diffusion, leakage, active transport). The process by which *c-erbB-2* protein becomes solubilized is also not fully understood and may account for a portion of the unexplained variability. Further investigation, currently underway, is exploring this line of inquiry. The association between salivary and serum *c-erbB-2* concentrations that were corrected by total protein concentrations was $r=0.39$; $p<0.001$.

Example 19

A relationship between serum *c-erbB-2* concentrations and serum CA 15-3 levels was found ($r=0.40$, $p>0.001$). This correlation was in agreement with the results reported by Krainer ($r=0.396$; $p>0.002$). When the serum *c-erbB-2* concentrations were corrected by total protein concentrations, the association between serum *c-erbB-2* concentrations and serum CA 15-3 levels was $r=0.36$, $p>0.001$.

Additionally, the data of previous examples also suggest that salivary *c-erbB-2* and serum *c-erbB-2* levels may be equivalent to salivary CA 15-3 and serum CA 15-3 levels as diagnostic markers (Figures 11, 15 and 16). The salivary and serum *c-erbB-2* concentrations were able to detect 87% and 94% of the subjects with cancer, respectively.

The salivary and serum *c-erbB-2* concentrations corrected by total protein detected 77% and 84%, respectively, of the subjects. This compares to 62% and 75% for the salivary and serum CA 15-3 marker. CA 15-3 levels were able to detect 75% of the malignant lesions at a 20 Units/ml cut-off value. The manufacturer recommended a 15 Units/ml cut-off value and indeed the sensitivity did increase to 97%; however, when this adjustment was made a sharp decline in specificity (35%) resulted as predicted by Stenman (1991). Conversely, when the cut-off value was increased to 40 Units/ml, the ability of the assay to detect cancer decreased to less than 30%. This is in agreement with the findings of Safi (1991) and Stenman (1991). Serum *c-erbB-2* levels, whether corrected for total protein or not, retained a margin of specificity at the 60% level for sensitivities over 90%.

Example 20

Information from the health questionnaire concerning age, race, tobacco usage, presence of systemic disorders, use of prescription medications, and menopausal status was also analyzed. These analyses confirmed the results from prior reports published by the investigators that these variables have no effect on salivary and serum *c-erbB-2* concentrations. Additionally, the findings for age (Watanabe, 1994), tobacco usage (Breuer, 1998), and menopausal status (Breuer, 1998) are supported by other studies; however, our study disagrees with Breuer (1998) which observed age related influences on marker concentrations. Breuer (1998) reported that among postmenopausal women, age was significantly related to *c-erbB-2* levels.

As a diagnostic medium, saliva has several biochemical advantages. Saliva is a clear, colorless liquid while serum may become milky when lipemic, red when blood cells are hemolyzed due trauma and icteric in the presence of liver disease. These color fluctuations in normal and disease altered serum can affect colorimetric assays such as ELISA, make it difficult to produce a consistent blank and interfere with the true values of the serum assay when compared to the consistent clarity of the assay standards. Since serum possesses more proteins than saliva assaying trace amounts of other factors (i.e., oncogenes, etc.), may result in a greater risk of non-specific interference and a greater chance for hydrostatic (and other) interactions between the factors and the abundant serum proteins.

From a logistical perspective, the collection of saliva is safe (i.e., no needle punctures), non-invasive and relatively simple, and may be collected repeatedly without discomfort to the patient.

The diagnostic benefits arising from the present invention could include the overall management of breast cancer in women. The diagnosis of breast cancer at an earlier stage allows a woman more choice in selection of various treatment options. A saliva based test would be useful in the postoperative management of cancer patients. Following tumor removal, an expected decrease in marker concentration should follow and eventually plateau to within a normal level indicating that the patient is free of disease. In contrast, a persistently high level of salivary markers may be indicative of tumor recurrence or persistence. Saliva could also be a cost effective method for monitoring the effectiveness of chemotherapy. Individuals should experience decreases in marker concentrations if the treatment regimen is effective.

While the principals of this invention have been described in connection with specific embodiments, it should be understood clearly that these descriptions, along with the chosen tables and data therein, are made only by way of example and are not intended to limit the scope of this invention, in any manner. For example, and without limitation, the methodology described herein can be extended to the diagnosis and monitoring of gall bladder, colon, rectal, pancreatic and oral cancers. Other advantages and features of this invention will become apparent from the following claims, with the scope thereof determined by a reasonable equivalents, as understood by those skilled in the art.

Claims:

1. A method of using a salivary biomarker to differentially diagnose carcinoma of the breast in a human test subject, said method comprising:
providing a salivary secretion specimen from a human subject to provide an individual salivary biomarker diagnostic for carcinoma of the breast, said biomarker soluble in said salivary secretion and selected from the group consisting of cancer antigen 15-3, tumor suppressor oncogene protein 53, oncogene c-erbB-2 and combinations thereof;
using the salivary secretion concentration of said individual biomarker to compare with a biomarker reference panel, said reference panel including biomarker constituents;
and
differentially identifying the diagnosis for said subject indicated by said comparison.
2. The method of claim 1 wherein said biomarker reference comprises a constituent panel developed using malignant tumor, benign tumor and control group populations.
3. The method of claim 1 wherein said individual biomarker is one constituent of a biomarker panel, said panel including at least one of cancer antigen 15-3, tumor suppressor oncogene protein 53 and oncogene c-erbB-2.
4. The method of claim 1 wherein said reference biomarker constituent panel includes value ranges for each said constituent.
5. The method of claim 3 wherein the presence of at least one of oncogene c-erbB-2 and proteinaceous expressions of said oncogene identifies the said subject having a malignant breast carcinoma.
6. The method of claim 3 wherein each said constituent is associated with a concentration value.

7. The method of claim 6 wherein said concentration of cancer antigen 15-3 is at least about 100% higher for said subject having a malignant breast tumor than said subject having a benign tumor.

8. The method of claim 6 wherein said concentration of oncogene protein 53 is at least about 25% lower for said subject having a malignant breast tumor than said subject having a benign tumor.

9. The method of claim 1 wherein said differential identification is an adjunct to a primary diagnostic method of testing said subject for carcinoma of the breast.

10. A post-operative method of monitoring the inhibition of tumor growth, said method comprising:

providing a human test subject, said subject post-operative to the removal of a malignant tumor;

providing a salivary secretion specimen from said subject to develop a post-operative biomarker panel, said panel having constituents selected from the group consisting of cancer antigen 15-3, tumor suppressor oncogene protein 53, oncogene c-erbB-2 and combinations thereof;

using said post-operative biomarker panel to compare with a pre-operative biomarker reference panel for said subject; and

determining the post-operative inhibition of breast malignancy by monitoring at least one constituent of said biomarker panels.

11. The method of claim 10 further including administering a chemotherapeutic regimen to said subject post-operatively.

12. The method of claim 11 wherein one said chemotherapy includes a therapeutic dose of cyclophosphamide, methotrexate and fluorouracil.

13. The method of claim 10 wherein said pre-operative and said post-operative panels include a c-erbB-2 biomarker constituent.

14. The method of claim 10 wherein said pre-operative and said post-operative panels include a tumor suppressor oncogene protein 53 biomarker constituent.

15. A method of using the concentration of an endogenously encoded protein to diagnose carcinoma of the breast, said method comprising:

providing a salivary secretion specimen from a human test subject to provide an individual protein biomarker diagnostic for carcinoma of the breast, said biomarker in said salivary secretion and selected from the group consisting of cancer antigen 15-3, tumor suppressor oncogene protein 53, oncogene c-erbB-2 and combinations thereof;

using said salivary secretion and said individual protein biomarker to compare with a reference protein concentration; and

determining an elevated concentration of said individual protein biomarker over said reference protein to diagnose said subject.

16. The method of claim 15 wherein said biomarker protein is one constituent of an individual biomarker panel.

17. The method of claim 15 wherein said biomarker protein is cancer antigen 15-3.

18. The method of claim 15 wherein said biomarker protein is an expression of oncogene c-erbB-2.

19. The method of claim 15 wherein said reference protein is developed for each of a malignant tumor, benign tumor and control group population.

20. The method of Claim 15 wherein said reference protein is one constituent of a panel.

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FIGURE 1

Medium	Status	CA 15-3 U/mg of protein	erb units/mg of protein	p53 pmol/mg of protein	CD pmol/mg of protein	EGFR fmol/mg of protein	Total Protein mg/ml
Saliva	Controls (n=15)	2.27±1.54	Not detectable	177.1 ±61.3	26.29±17.22	1.03 ±0.69	1.25±0.82
	Benign (n=8)	2.22±1.95	Not detectable	180.7 ±70.78	40.57±13.05	0.37 ±0.31	1.44±0.92
	Ca in situ (n=12)	5.26±4.12 [†]	51.3±43.96 [†]	134.6 ±63.8	34.5±27.95	0.92 ±0.8	1.71±0.79
Serum	Controls (n=15)	16.17 ±4.64	Not detectable	14.2±7.9	67.5 ±35.61	8.5 ±8.45	24.54±13.86
	Benign (n=8)	15.25±5.04	Not detectable	14.63 ±8.8	45.0±20.98	3.52 ±1.89	27.33±14.23
	Ca in situ (n=12)	24.68±10.9 ^{††}	81.68±111.77 ^{††}	8.9 ±7.7	63.17±38.4	5.63 ±3.10	29.23 ±11.08

FIGURE 2

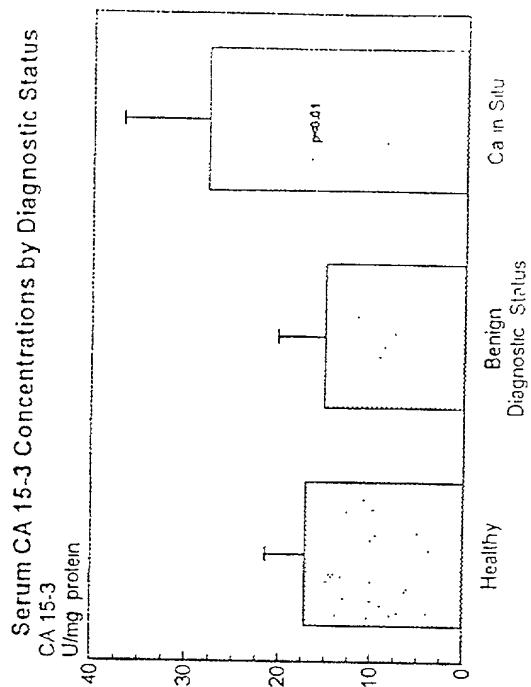
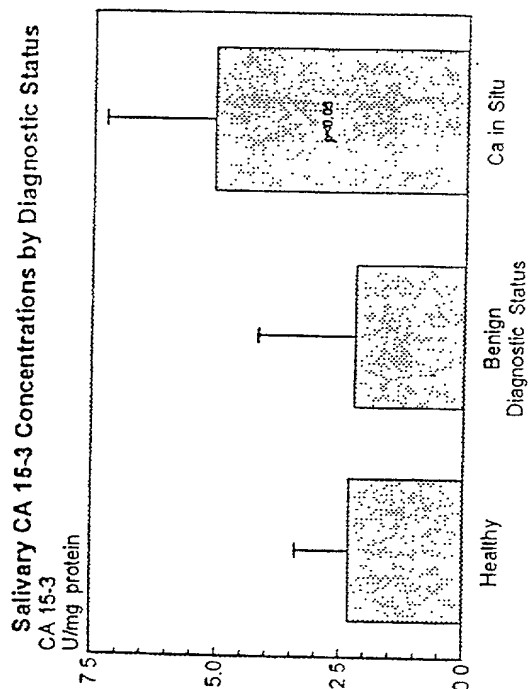
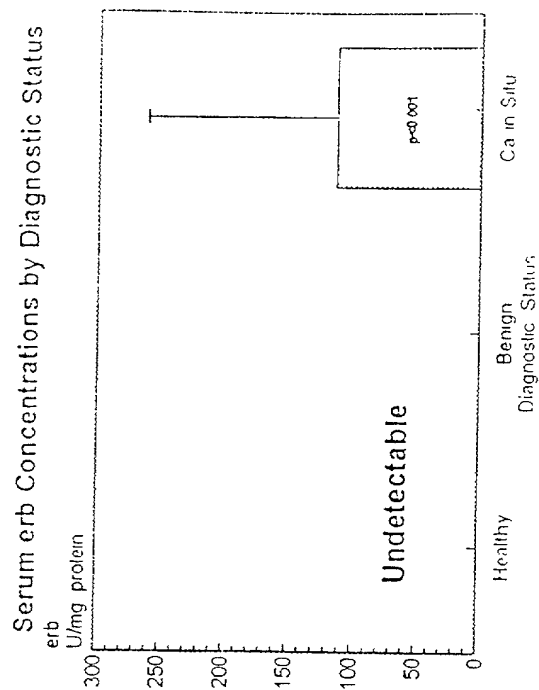
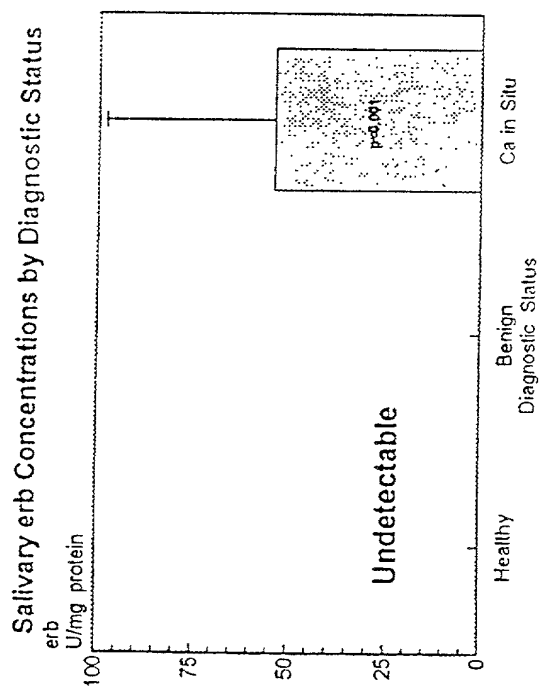


FIGURE 3



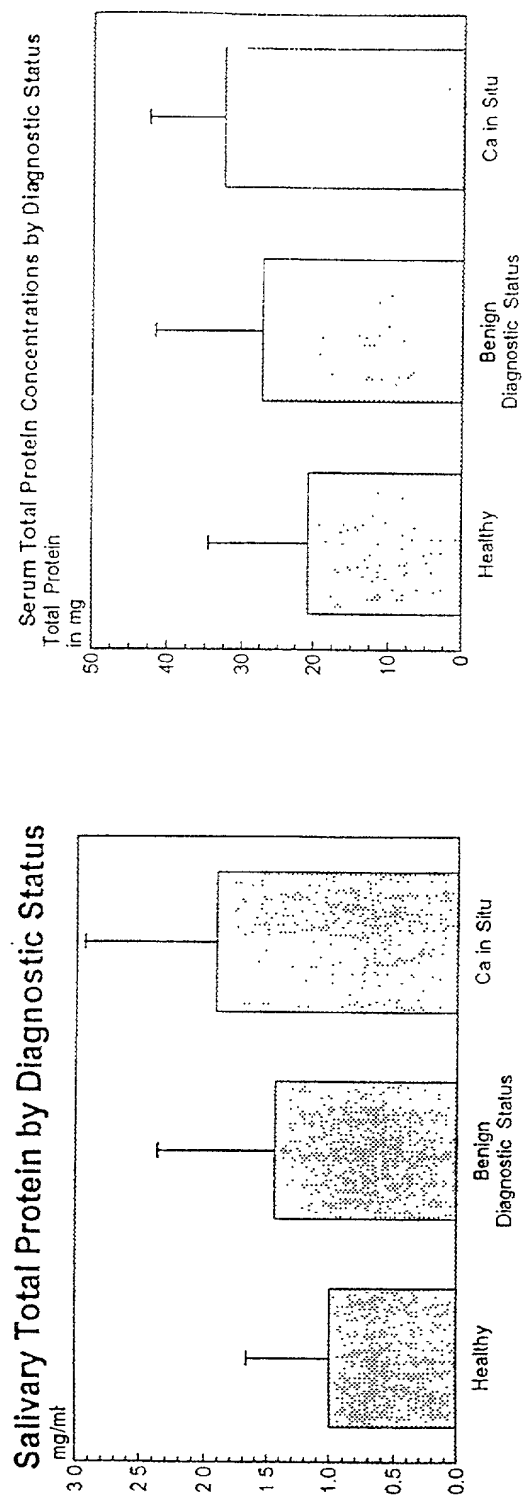
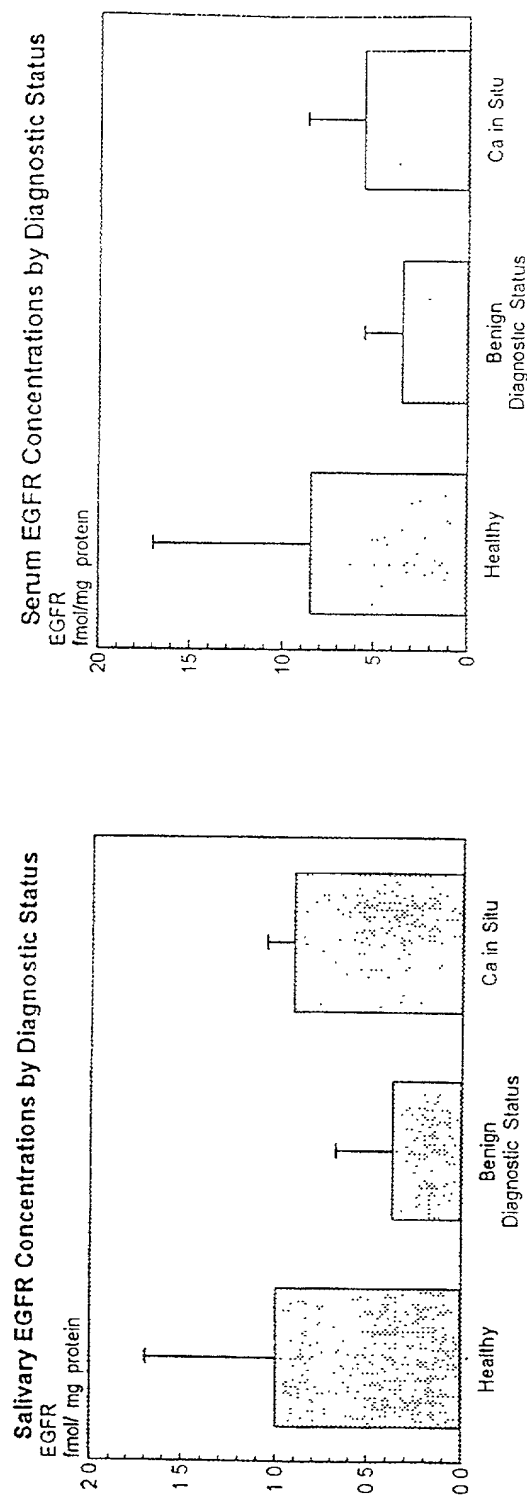


FIGURE 6

FIGURE 6

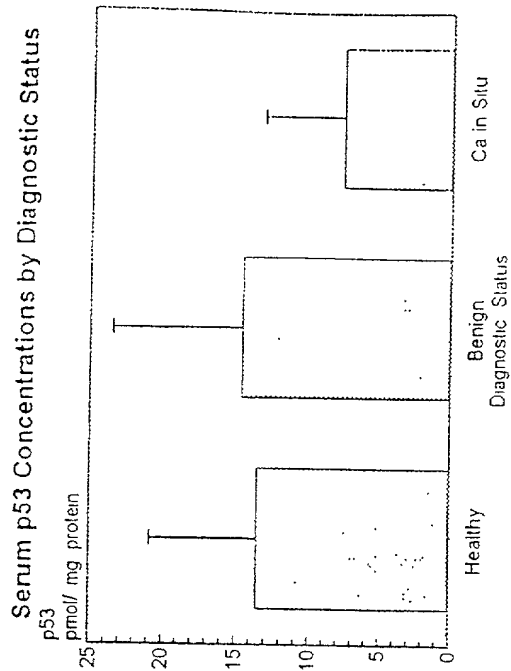
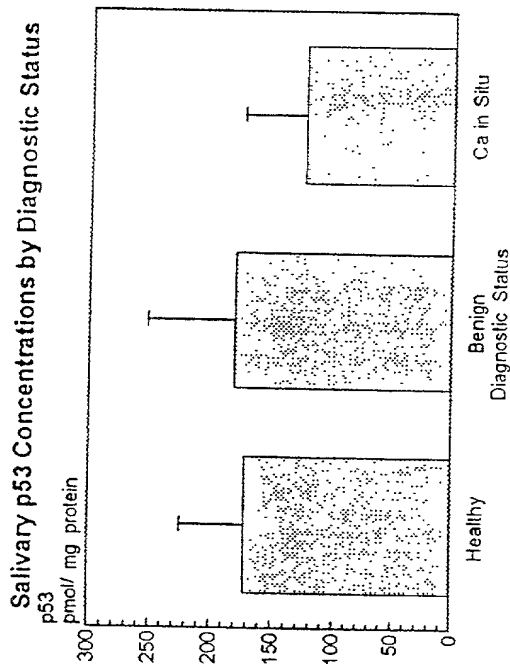


FIGURE 7

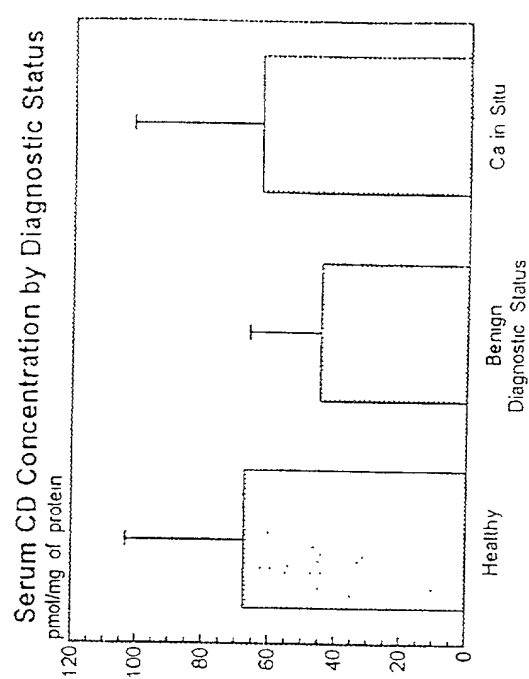
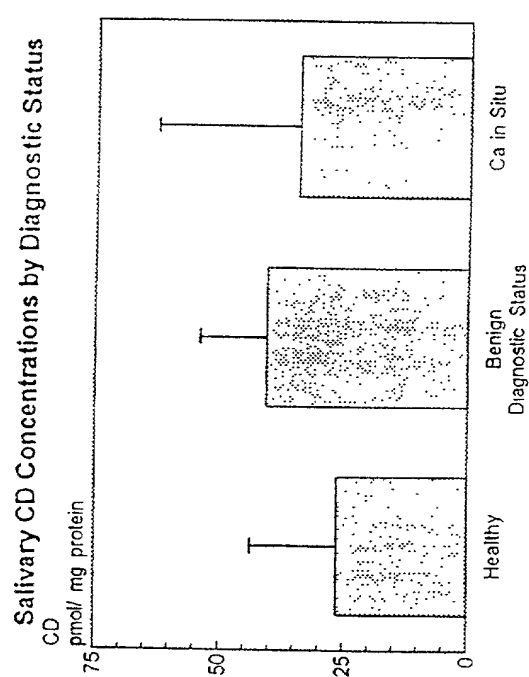


Figure 8

Mean and Standard Error of the Mean (S.E.M.) Values for c-erbB-2									
Characteristics	Healthy Controls			Benign Tumors			Malignant Tumors		
	n	age	Mean (S.E.M.)	n	age	Mean (S.E.M.)	n	age	Mean (S.E.M.)
Saliva c-erbB-2	57	39.6	94.70 (± 8.39)	41	45.0	99.72 (± 10.94)	30	50.8	222.22 (± 19.22)
Saliva c-erbB-2 (Units/mg protein)	57	39.6	71.13 (± 6.07)	41	45.0	63.75 (± 7.00)	30	50.8	143.58 (± 11.53)
Salivary Protein (mg/ml)	56	39.3	1.48 (± 0.09)	41	45.0	1.58 (± 0.08)	30	50.8	1.67 (± 0.10)
Salivary CA 15-3 (Units/ml)	42	38.0	3.19 (± 0.52)	33	43.9	7.23 (± 1.58)	30	50.8	10.90 (± 3.44)
Salivary Flow Rates (ml/min.)	56	39.6	1.90 (± 0.12)	41	44.3	1.48 (± 0.12)	30	50.8	1.15 (± 0.13)
Serum c-erbB-2	57	39.6	1472.15 (± 78.78)	41	44.3	1515.63 (± 155.48)	30	50.8	3490.67 (± 364.48)
Serum c-erbB-2 (Units/mg protein)	57	39.6	28.04 (± 0.63)	41	44.3	33.05 (± 3.44)	30	50.8	85.97 (± 9.31)
Serum CA 15-3 (Units/ml)	42	38.0	10.88 (± 0.74)	33	43.9	15.91 (± 0.88)	30	50.8	41.49 (± 10.65)
Serum Protein (mg/ml)	56	39.3	53.04 (± 1.41)	44	44.3	42.90 (± 1.16)	30	50.8	42.15 (± 1.58)

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Figure 9

Frequency Distribution for Questionnaire Data				
Characteristic	Controls	Benign Tumors	Malignant Tumors	p-value
n	57	44	30	--
Tobacco Usage	--	--	--	2.40E-06
Yes	3	15	16	--
No	54	29	14	--
Race	--	--	--	0.00063
Caucasian	38	13	12	--
African - American	19	31	18	--
Systemic Diseases	--	--	--	0.334
Yes	16	17	10	--
No	41	23	20	--
Prescribed Medications	--	--	--	0.449
Yes	25	23	16	--
No	32	18	14	--
Menopausal Status	--	--	--	0.00016
Premenopausal	40	19	9	--
Perimenopausal	0	10	17	--
Postmenopausal	17	15	4	--

Figure 10

Mean and Standard Error of the Mean (S.E.M.) Values Values for c-erbB-2 Among Varying Malignant Tumor Stages					
Characteristic		Saliva		Serum	
Clinical T Stage	n	c-erbB-2 Units/ml Mean (S.E.M.)	c-erbB-2 Units/mg protein Mean (S.E.M.)	c-erbB-2 Units/mg protein Mean (S.E.M.)	c-erbB-2 Units/mg protein Mean (S.E.M.)
	7	211.15 (± 25.91)	133.75 (± 10.27)	3428.13 (± 564.29)	95.57 (± 16.83)
	16	190.50 (± 19.36)	137.01 (± 17.85)	3680.15 (± 605.42)	89.32 (± 14.60)
Mean and Standard Error of the Mean (S.E.M.) Values Values for c-erbB-2 Among Node Positive and Node Negative Cancer Patients					
Tumor Sizes	n	c-erbB-2 Units/ml Mean (S.E.M.)	c-erbB-2 Units/mg protein Mean (S.E.M.)	c-erbB-2 Units/ml Mean (S.E.M.)	c-erbB-2 Units/mg protein Mean (S.E.M.)
	16	192.70 (± 21.02)	119.52 (± 12.74)	2296.36 (± 308.42)	87.44 (± 8.83)
	7	227.25 (± 14.36)	192.42 (± 29.19)	5308.55 (± 2066.35)	126.63 (± 49.54)

Figure 11

Cutoff Values, Sensitivity, Specificity, and Percent Area Under Curve for Salivary and Serum c-erbB-2 and CA 15-3 Concentrations					
Characteristics	Cutoff Value	Sensitivity	Specificity	Percent Area Under Curve	
Saliva c-erbB-2	110	0.87	0.65	75.7	
Saliva c-erbB-2 (Units/mg protein)	100	0.77	0.77	76.7	
Salivary CA 15-3	4	0.62	0.79	70.5	
Serum c-erbB-2	2000	0.94	0.60	76.7	
Serum c-erbB-2 (Units/mg protein)	50	0.84	0.68	76.0	
Serum CA 15-3	20	0.75	0.44	62.2	

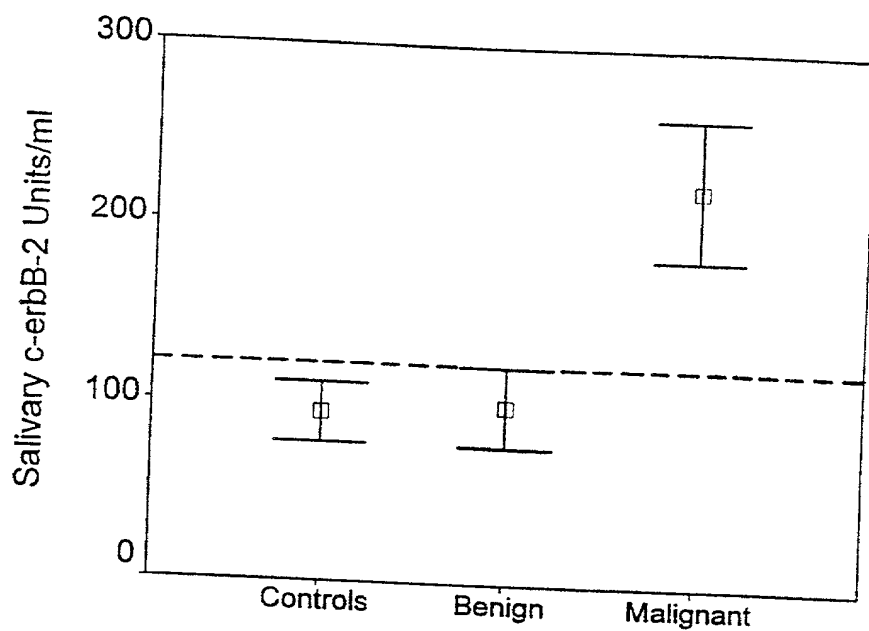


Figure 12a

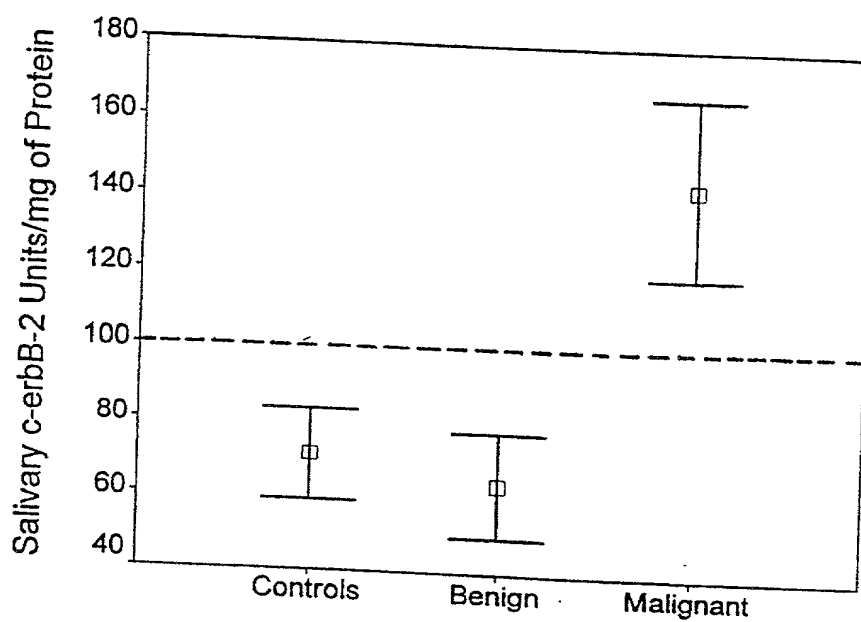


Figure 12b

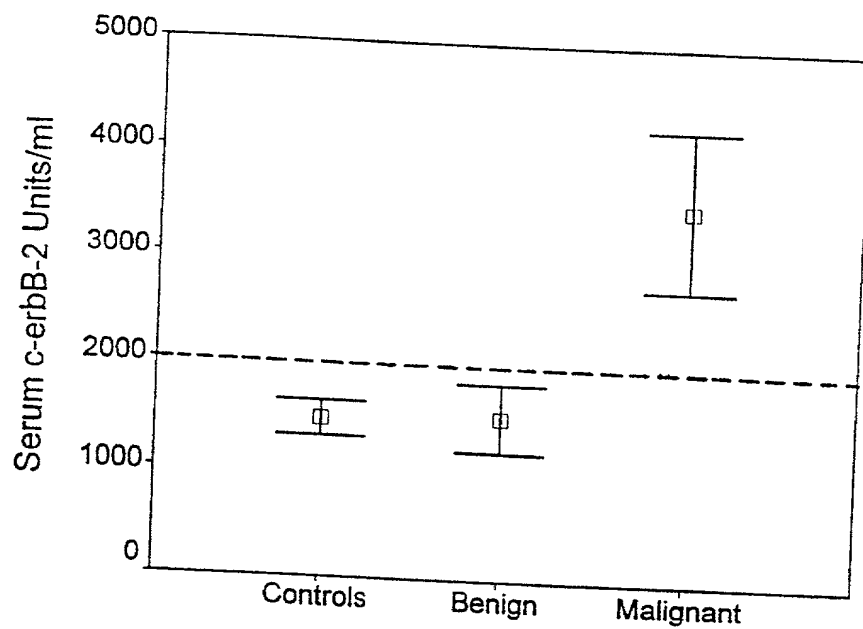


Figure 13a

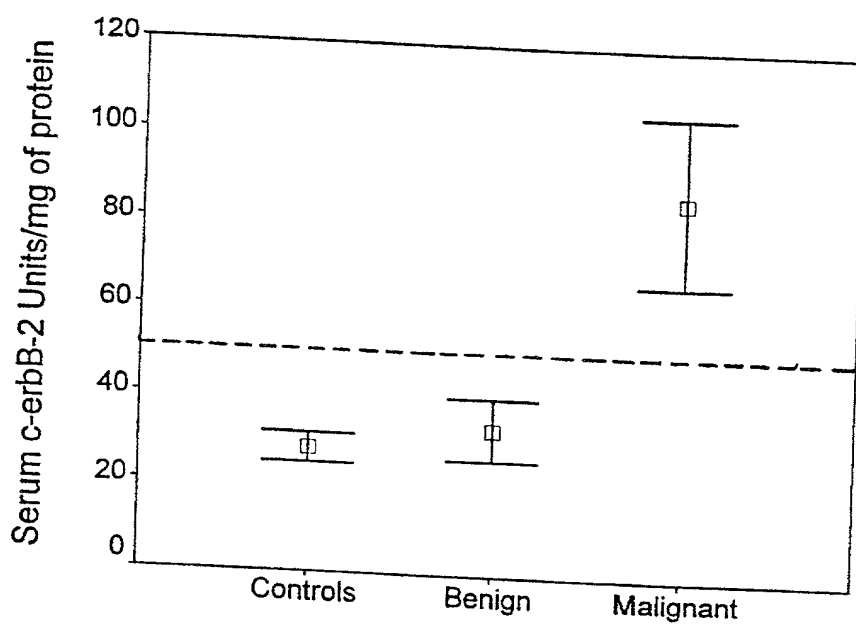


Figure 13b

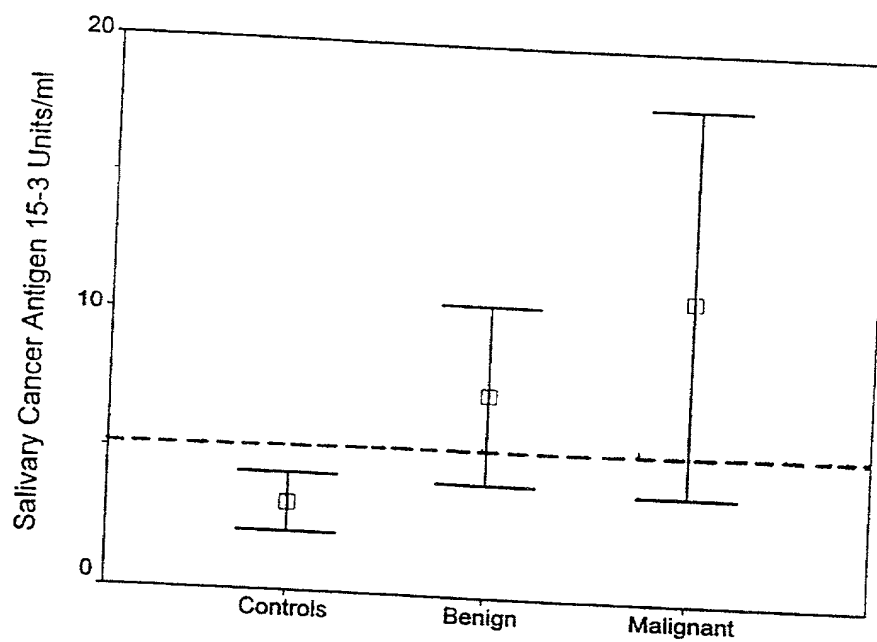


Figure 14a

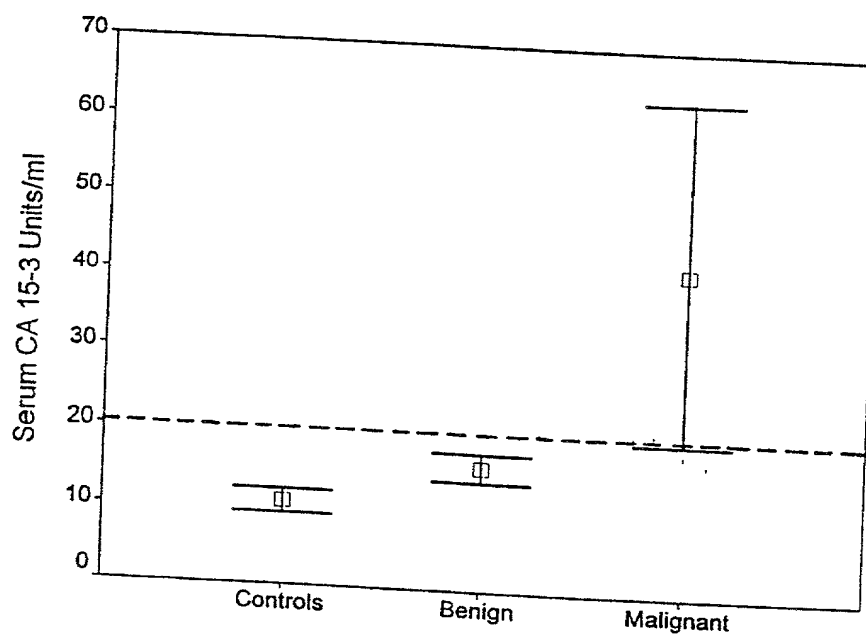


Figure 14b

Figure 15

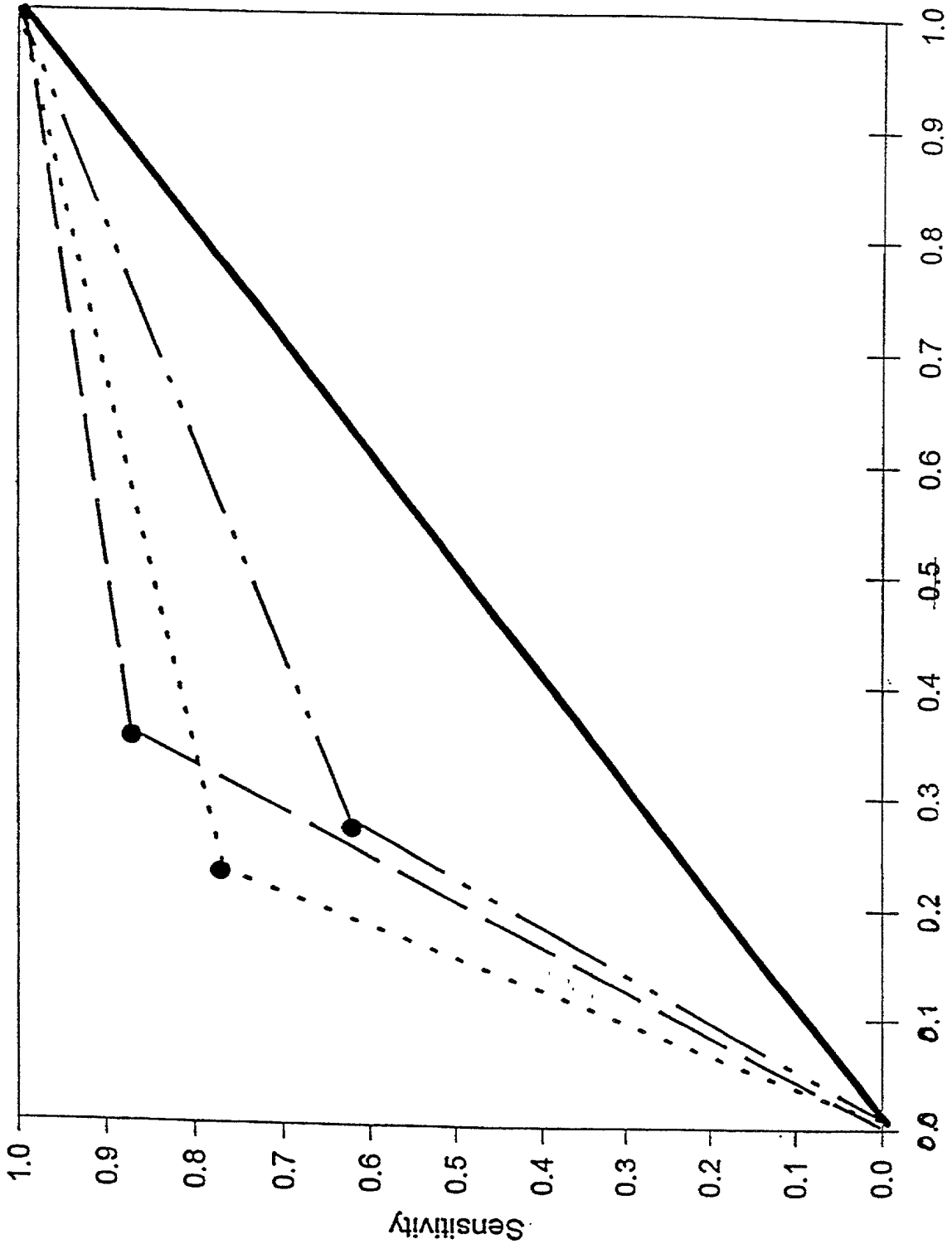
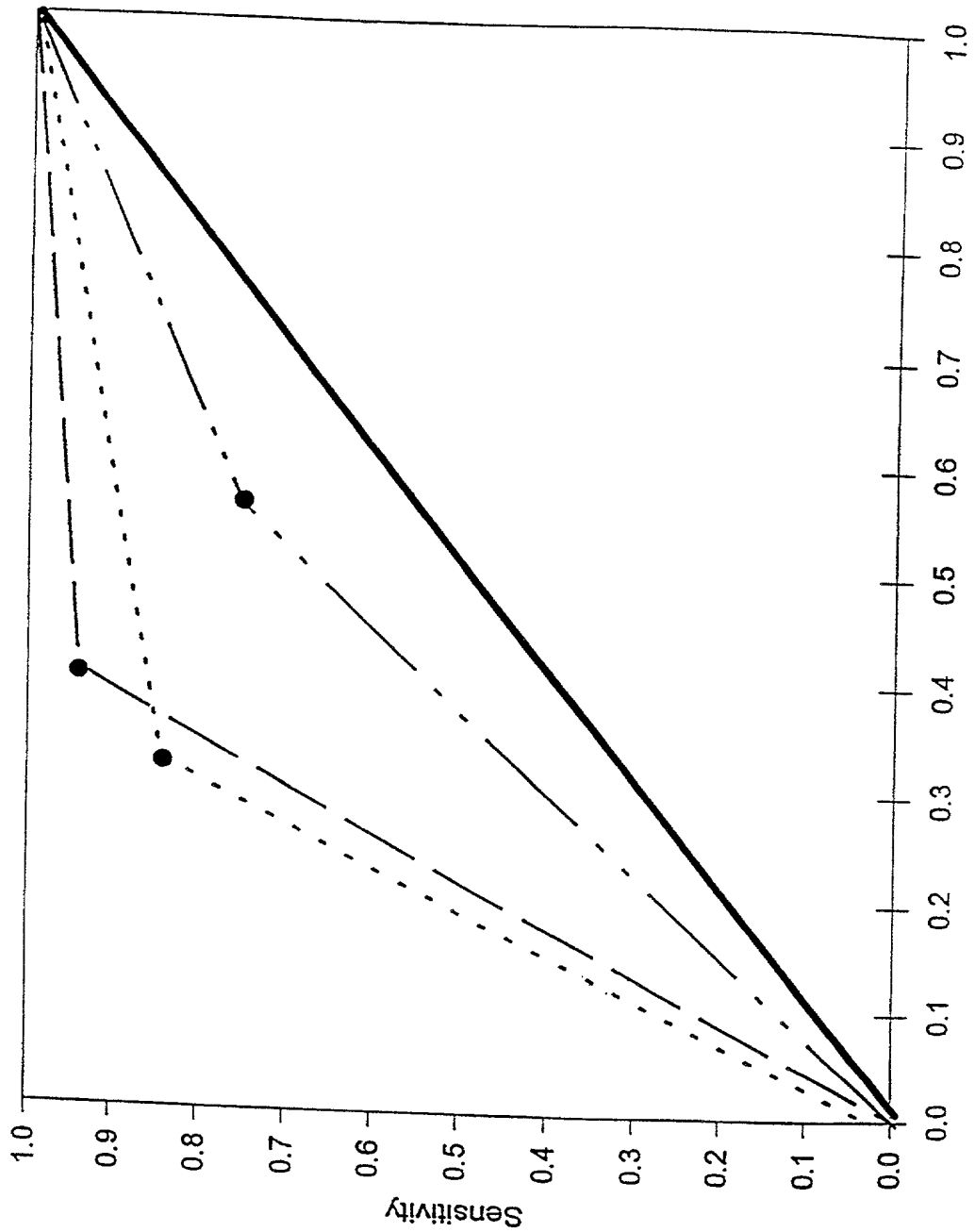


Figure 16



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PTO/SB/01 (12-97)

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DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)	Attorney Docket Number	4856-CIP
	First Named Inventor	Charles F. Streckfus
	COMPLETE IF KNOWN	
	Application Number	09/914,501
	Filing Date	
<input type="checkbox"/> Declaration Submitted with Initial Filing	OR	<input checked="" type="checkbox"/> Declaration Submitted after Initial Filing (surcharge (37 CFR 1.16 (e)) required)
	Group Art Unit	
	Examiner Name	

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

METHOD OF DIAGNOSING AND MONITORING MALIGNANT BREAST CARCINOMAS

the specification of which (Title of the Invention)

☐ is attached hereto
OR

☒ was filed on (MM/DD/YYYY) 08/29/2001 as United States Application Number or PCT International

Application Number 09/914,501 and was amended on (MM/DD/YYYY) 08/29/2001 (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached? YES	NO
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☐ Additional foreign application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto:

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)

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[Page 1 of 2]

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I hereby claim the benefit under 35 U.S.C. 120 of any United States application(s), or 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application or PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number (if applicable)
09/259,993 PCT/US00/05364	1 March 1999 1 March 2000	6,294,349

☐ Additional U.S. or PCT international application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

As a named inventor, I hereby appoint the following registered practitioner(s) to prosecute this application and to transact all business in the Patent

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☒ Registered practitioner(s) name/registration number listed below

Name	Registration Number	Name	Registration Number

☐ Additional registered practitioner(s) named on supplemental Registered Practitioner Information sheet PTO/SB/02C attached hereto

Direct all correspondence to: ☒ Customer Number
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OR ☐ Correspondence address below

22922

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of Sole or First Inventor:

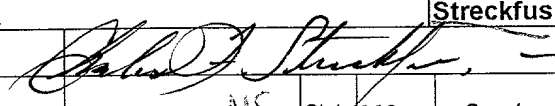
☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])

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Inventor's Signature  Date 12/7/01

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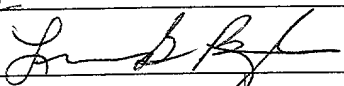
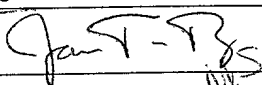
☒ Additional inventors are being named on the 1 supplemental Additional Inventor(s) sheet(s) PTO/SB/02A attached hereto.

PTO/SB/02A (11-00)

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Supplemental Sheet
Page 1 of 1

Name of Additional Joint Inventor, if any:		<input type="checkbox"/> A petition has been filed for this unsigned inventor	
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Mailing Address			
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